PB-213 120

ENTERIC BACTERIAL DEGRADATION OF STREAM DETRITUS

Charles W. Hendricks

Georgia University
Athens, Georgia

December 1971

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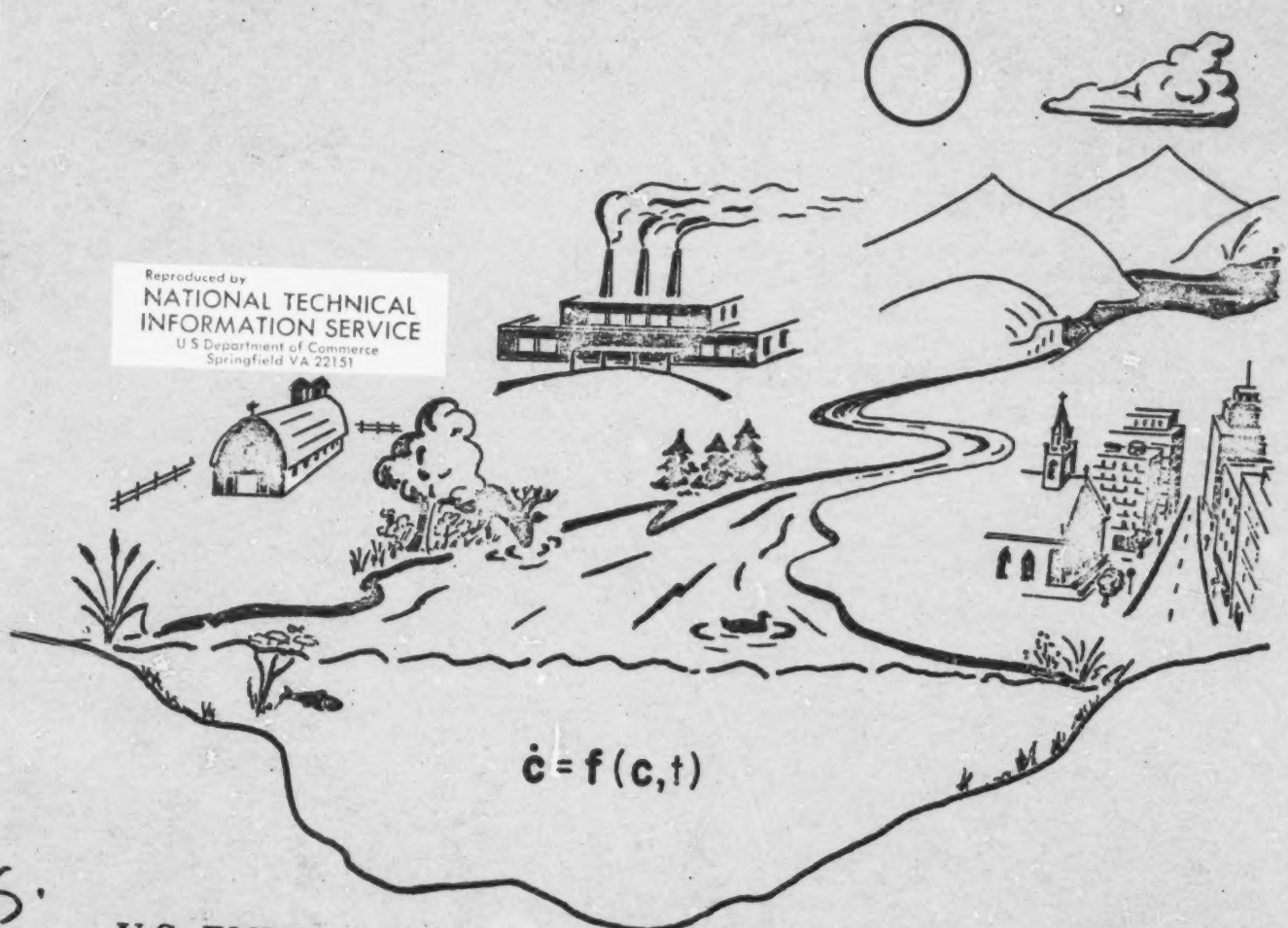

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SELECTED WATER RESOURCES ABSTRACTS
INPUT TRANSACTION FORM

1. Report No.

2.

3. Accession No. W. 73-01783 ~~72-14794~~

4. Title: ENTERIC BACTERIAL DEGRADATION OF STREAM DETRITUS,

5. Report Date

6.

7. Author(s): Hendricks, C[redacted] W.

8. Performing Organization Report No.

9. Organization: Georgia Univ., Athens, Dept. of Microbiology

10. Project No. EPA Grant 16050 EQS 12/71

11. Contract/Grant No.

12. Sponsoring Organization: Environmental Protection Agency, Water Pollution Control Research Series [redacted], December 1971. 105 p. 24 fig., 33 tab., 58 ref. EPA 16:16050 EQS 12/71

13. Type of Report and Period Covered

15. Supplementary Notes

16. Abstract

To relate basal nutrients in the water and on the bottom of a warm, fresh water stream to their ability to support growth and multiplication of pathogenic and nonpathogenic enteric bacteria, three studies were designed including a river water quality analysis, respiration experiments, and continous culture experiments. The presence of coliform bacteria primarily not producing intestinal disease, serves as indicators for potentially infectious microorganisms. Any growth by either coliform bacterial group or the disease producing organisms in the natural aquatic environment could significantly alter our present concepts of detection and surveillance of these organisms. Tests in detecting and analyzing polluted waters, and from levels of the self-purification potential, showed that Oconee River, a typically non-polluted stream of the North Georgia piedmont, is capable of supporting bacterial growth including coliforms. Both organic and inorganic compounds can be adsorbed by bottom sediments and some, after removal, can be used as food for both coliform and intestinal disease producing bacteria. If intestinal pathogenic bacteria such as Salmonella, Shigella, and Arizona can reach our streams and multiply, chances of human disease from the untreated or partially treated water are increased. (Jones-Wisconsin)

17a. Descriptors: *Enteric bacteria, *Degradation (Decomposition), *Streams, *Detritus, Nutrients, Bottom sediments, Water quality, Water pollution effects, Pathogenic bacteria, Bacteria, Salmonella, Coliforms, Shigella, Public health, E. coli

17b. Identifiers: North Oconee River (Ga.), Bdellovibrio bacteriovorus, Enterobacter aerogenes

17c. COWRR Field & Group: 05C

Copy available from GPO $.5 Doc [illegible], $1.00; microfiche from NTIS as PB 213 120, $0.95.

18. Availability

19. Security Class. (Report)

20. Security Class. (Page)

21. No. of Pages: -10-

22. Price

Send To:
WATER RESOURCES SCIENTIFIC INFORMATION CENTER
U.S. DEPARTMENT OF THE INTERIOR
WASHINGTON, D. C. 20240

Abstractor: E. S. Jones va

Institution: Wisconsin Univ., Madison

ENTERIC BACTERIAL DEGRADATION OF STREAM DETRITUS

by

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for the

OFFICE OF RESEARCH AND MONITORING

ENVIRONMENTAL PROTECTION AGENCY

Project # 16050 EQS

December, 1971

For sale by the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402 - Price $1.00

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ABSTRACT

A laboratory and field investigation was conducted between August, 1968 and July, 1971 to relate basal nutrients in the water and on the bottom of a warm, fresh water stream to their ability to support the growth and multiplication of pathogenic and nonpathogenic enteric bacteria. Three independent studies, including (1) a water quality analysis of the Oconee River (Clark County, Georgia), (2) respiration experiments, and (3) continuous culture experiments were designed to provide useful information in this research.

The results of this investigation indicate that natural populations and selected laboratory strains of enteric bacteria have the capacity to metabolize substrates that were present in the Oconee River environment including autoclaved river water. These organisms, however, lacked the ability to increase in numbers in continuous culture with river water and suspended detritus recovered above a secondary sewage treatment facility, but they did demonstrate positive growth rates with substrates recovered below the plant.

Data from this study also demonstrated that the sands and clays forming the stream bottom have the capacity to sorb substrates from the overlayering water, and that sediment eluates will stimulate the respiration rate of the study bacterial strains. These results suggest that the stream bottom can provide a suitable environment for the growth of bacterial species and perhaps control basal nutrient concentration in the water itself.

This report was submitted in fulfillment of Project Number 16050 EQS, under the sponsorship of the Environmental Protection Agency.

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SECTION I

CONCLUSIONS

1. Based upon water quality determinations for basal nutrients and types and numbers of bacteria present, the North Oconee River above Athens, Ga. does not appear grossly contaminated. However, the Bailey St. sewage plant does contribute significantly to the basal nutrient concentration and an unknown number of Salmonella to the stream that is subsequently used for recreational purposes.

2. There appears to be more Salmonella associated with the bottom sediments than are present in the overlaying water in the area below the sewage plant effluent (Site 3).

3. The presence of an inducible formic hydrogenlyase system at 44.5 C in fecal coliforms (E. coli) was found to be the basis for the Eijkman fecal coliform concept.

4. The laboratory strains of enteric bacteria utilized in this study could metabolize substrates present in river water, collected detritus and extracts of bottom sediments.

5. The test bacterial strains were unable to grow in chemostats when river water taken above the sewage plant was employed. River water taken below the sewage plant effluent did support the growth of these organisms.

6. Although the observed multiplication rates for the enteric bacteria used in the study were small in comparison to that which can be achieved in laboratory media, any process which would have an end result of contributing oxidizable substrates to the system could in effect substantially raise these growth rates.

7. Bdellovibrio bacteriovorus, an obligate bacterial parasite for Gram negative bacteria, including Escherichia coli, can infect the host in river water, but it is doubtful that the parasite is a major factor in stream self-purification mechanisms.

8. Whether or not enteric bacteria grow in substrates from the aquatic environment seems to be a function of the integrated environmental parameters such as basal nutrient concentration, temperature, stream flow, etc. That is, the concept of a growth limiting nutrient for enteric bacteria may not be valid in a relatively non-polluted stream.

SECTION II

RECOMMENDATIONS

This research project was limited to laboratory experiments using natural substrates recovered from the Oconee River in attempts to determine if they could be metabolized by enteric bacteria including pathogenic species. The river water, sediment eluates, and processed detritus substrates used in this study did receive mild laboratory treatment prior to use, and it is recommended that in situ studies of a similar nature be made before data in this report is extrapolated directly to the natural aquatic environment.

Based upo the data in this report and taking into consideration the above statement on extrapolation of data to the environment, certain general recommendations, however, can be made.

1. Bottom sediments should be included in routine sampling procedures, especially during water quality surveys.

2. Depending upon the immediate use of the water and the area in question, provisions should be made to monitor water quality prior to any disruption of the integrity of the sediment layer of a lake, stream or estuary. Parameters that would be of importance and perhaps predict problems of a public health nature are: a) presence and numbers of specific organisms and b) a measure of microbial activity such as respiration determinations.

3. Work is needed to determine the significance of the growth of potentially dangerous organisms in fresh water. Questions that would be of interest include: a) are other organisms besides enteric capable of growth in fresh water, b) are such organisms virulent, c) are toxic factors synthesized in quantity to be hazardous and d) under what conditions could such growth be controlled.

4. Further work is also needed to elucidate the observed sediment-nutrient interaction and to determine how this interaction affects the microbiological quality of water.

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SECTION III

INTRODUCTION

The principles governing the growth and multiplication of bacteria in nutritionally adequate media at near optimal temperatures are well known. In general, a few bacterial cells can produce vast populations in these media since the initial concentration of nutrients does not become a critical factor for growth. However, in natural environments, or in media where nutrient concentrations are marginal and temperatures far from optimal, multiplication of mesophilic bacteria often becomes erratic, and precise growth rates are not easily determined. A few investigators, including Kusnezow (1959) and Ruttner (1964), are convinced that many heterotrophic bacterial species are capable of growth in fresh water lakes and streams. These bacteria seem to be involved in organic dissimilation processes in which complex organic compounds are returned to the biotope as inorganic and relatively simple organic substance for recycling by photo- and chemosynthetic organisms.

Certainly, nutritional conditions present in the aquatic environment are of great importance as determinants of whether or not a particular bacterial species will survive and later multiply. Probably only a very small fraction of the total bacterial growth occurs in the free flowing water where an adequate supply of nutrients is not easily obtained. However, on, or in, the bottom sediments, extensive growth of bacteria can occur in the micro-environment where nutrients can be in high concentration surrounded by a relatively vast area devoid of nutrients. Temperatures in this environment would not fluctuate as sharply as the diurnal variations of the stream proper, thus creating more stable conditions.

This study includes attempts to relate the types of organic material in and on the bottom of a warm, fresh water stream to their ability to support the growth and multiplication of pathogenic and nonpathogenic enteric bacteria. Once specific knowledge has been obtained concerning the utilization of this material, we would be in a better position to understand the roles of both the aquatic environment and the bacteria in a polluted stream. Such data could also aid in the classification of rivers and streams and form a foundation for the construction of a model system which might be able to predict the theoretical pollution limits of a given body of water.

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Previous investigations concerning the nutritional requirements for enteric bacteria, especially for E. coli, have contributed greatly to our understanding of the organism and its metabolism, but most of this knowledge has been derived with the use of artificial culture media containing organic materials in concentrations and in type far exceeding those found in natural surface waters and bottom sediments. It is conceivable that present knowledge concerning well-nourished bacteria may not be applicable to studies dealing with microbial growth and reproduction in aquatic environments, especially with bacteria associated with pollution, and those which are not indigenous to the environment.

Many intestinal disease-producing bacteria in man and higher animals, including those within the family Enterobacteriaceae, are not fastidious in their nutritional requirements. In fact, many of the prototrophic species belonging to the genera Proteus, Salmonella, Paracolobactrum, and Shigella are able to grow and reproduce in a basic inorganic salts medium similar to that used to grow algae, providing a suitable carbon source is present. McGrew and Mallette (1962) have shown that intestinal bacteria can reproduce in such a medium that contained less than 5 μg/ml of glucose, and this concentration approaches that of hexoses commonly found in unpolluted river water.

If potentially pathogenic bacteria can obtain adequate nutrients in the natural aquatic environment and are also capable of reproduction, needed information could be obtained on mesophilic growth in natural, low nutrient substrates at temperatures far removed from their optimum 35-47 C range. Such research could also aid in the bacteriological classification of waters capable of becoming polluted if dilution purification factors were overcome and may clarify present ideas concerning stream self-purification and the role of aquatic environments in the dissemination of disease.

This investigation consists of three independent studies each of which was designed to provide useful biochemical and bacteriological information concerning the ability of enteric bacteria to survive and multiply in the presence of low levels of natural aquatic substrates. These studies are as follows:

1. Water quality analysis of the North Oconee River at three selected sites at Athens, Georgia.
2. Evaluation of the ability of enteric bacteria to use natural aquatic substrates by oxygen uptake experiments.
3. Evaluation of the ability of enteric bacteria to use natural aquatic substrates by continuous culture experiments.

SECTION IV

METHODS AND MATERIALS

Part 1

Water Quality Analysis of the North Oconee River at three selected sites at Athens, Georgia

Chemical and bacterial assays

Site selection: Three sites were selected on the north branch of the Oconee River that flows through Athens, Georgia (Fig. 1). Site one is situated about 4 mi. northeast of the city and the river in this area has been flowing through agricultural and undeveloped land (Fig. 2). Site two is located near the center of Athens and well within the city limits. The river at this point has received water from various storm drains and creeks that drain the residential areas. No domestic sewage is known to be added to the river in this area (Fig. 3). Site three is situated about 750 meters below one of the two Athens municipal sewage plants (Fig. 4). The sewage at this facility receive both primary and secondary treatment, and the effluent is chlorinated and allowed to settle prior to addition in the stream.

Sample collection and analysis: Water, sediments, and stream detritus in various forms were collected by the techniques of Morrison and Fair (1966) and returned to our laboratories for substrate and bacteriological analysis. Specific parameters of interest are types and numbers of bacteria, water temperature, protein, orthophosphate, ammonia nitrogen, and carbohydrate content, pH, and Carbon-Nitrogen-Hydrogen ratios. These determinations were made on at least a biweekly basis and also when water was collected for a particular growth study.

Assay procedures: Chemical and physical parameters of the Oconee River were monitored by techniques approved by the American Public Health Association and published in Standard Methods for the Examination of Water and Wastewater (APHA, 1971). Glucose and total hexose were measured by the Glucostat (Worthington Biochem. Corp.) and anthrone (Morris, 1948) procedures, respectively, and protein content was determined by the Folin-Ciocalteau and Biuret procedures (Colowick and Kaplan, 1955). Enteric bacteria enumerations were made according to the techniques of Morrison and Fair (1966), and species identification made with the use of Bergey's Manual (Breed et al., 1957), the taxonomic aid, The Identification of Enterobacteriaceae

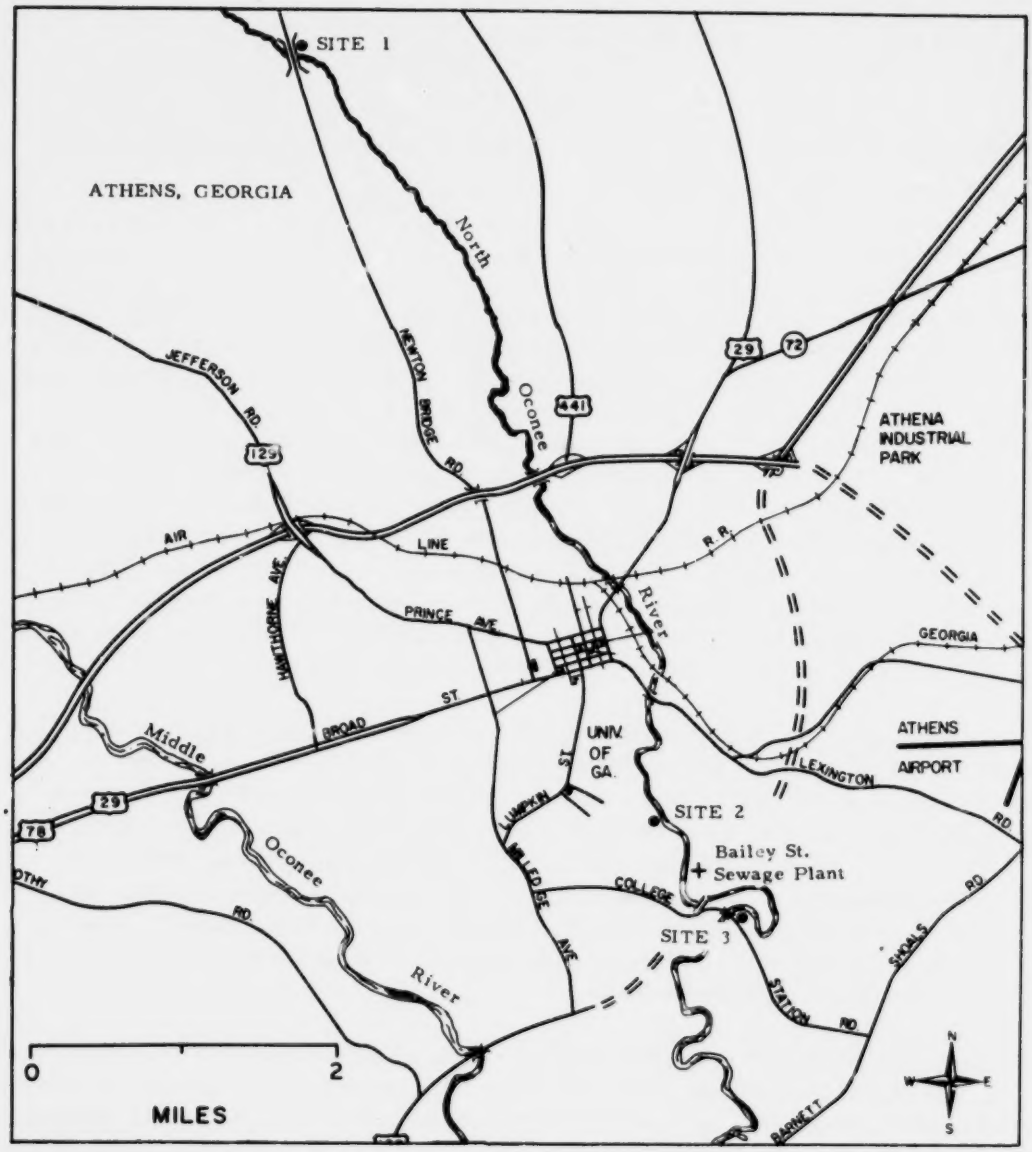


Fig. 1. Map of Athens, Georgia showing the three study sites.

Fig. 2. Study site 1.

Fig. 3. Study site 2.

Fig. 4. Study site 3.

(Edwards and Ewing, 1962) and then confirmation by NCDC, Atlanta, Ga.

Sampling procedure evaluations

Two separate field studies were initiated to evaluate the reliability of the Eijkman fecal coliform concept and the recovery of salmonellae in stream bottom sediments vs. surface waters.

Formic hydrogenlyase induction as a basis for the Eijkman fecal coliform concept: There have been numerous attempts to differentiate between a "fecal" and a "non-fecal" coliform, and the most successful of these are those of the elevated temperature of incubation variety (Geldreich, 1966.) These procedures are primarily modifications based upon the Eijkman (1904) observation that members of the Escherichia group, as described by Parr (1938a, b), could produce gas in a glucose medium at 46 C, whereas Enterobacter (Aerobacter) could not. Perry and Hajna (1944) improved upon the Eijkman technique by substituting lactose for glucose, adding bile salts and a buffering system to the medium and lowering the incubation temperature to 44.5 C. Utilizing modification of this basic fecal coliform procedure, Geldreich et al. (1962), and more recently Mishra et al. (1968), have observed a positive correlation of gas production at the elevated incubation temperature with Parr's Escherichia group of coliforms. More significantly, however, Mishra et al. found that more than 52% of the Enterobacter types recovered also gave positive elevated temperature reactions.

The basic mechanism by which gas is produced by Escherichia and not by Enterobacter at elevated temperature is not clearly understood, nor is it known with certainty which Enterobacter strains from aquatic or terrestrial environments are capable of producing gas at these temperatures. The present investigation was undertaken to determine which particular gas-producing enzyme system in Enterobacter is affected by the incubation at 44.5 C and to test the basic concept of the Eijkman procedure with known coliform strains and organisms recovered from the aquatic environment.

Basic Warburg Procedure: The activity of induced formic hydrogenlyase was determined in a Warburg respirometer (Aminco) by the technique of Quist and Stokes (1969). Duplicate vessels contained 2.0 ml of a washed cell suspension in the main compartment, and 0.2 ml of a 20% KOH solution in the center well for the absorption of CO_2. Substrates for induction included in the side arms were 60 µmoles of sodium formate (0.3 ml), 20 µmoles of glucose (0.2 ml), and 0.1 ml of a 10% concentration

of yeast extract (Difco). To ensure that the enzyme system was being induced in the Warburg vessels and was not preformed in the original culture flasks, 0.03 mg of chloramphenicol (0.3 ml) was added to one of the Warburg vessels in each series to inhibit de novo protein synthesis. The remaining flask received 0.3 ml of 0.1 M phosphate buffer (pH 6.9). The gas phase in each experiment was N_2, and the bath temperature was at either 30.0 or 44.5 C, depending on the particular experiment. Each vessel was incubated with shaking until a constant rate of H_2 evolution was reached, and results were expressed in $Q(H_2)$ values, as microliters of H_2 evolved per hour per milligram of dry cells. There was virtually no endogenous H_2 production and no H_2 formed in control vessels containing only cells and sodium formate.

Selection of organisms: Enteric bacteria employed in this study were obtained from both the American Type Culture Collection (Rockville, Md.) and by recovery from the North Oconee River in Clark County, Ga. River water samples were collected by techniques suggested by the American Public Health Association (1966) and cultured on M-Endo-MF Broth (BBL) by the use of the membrane filter procedure. After incubation at 44.5 ± 0.1 C for 16 to 18 hr, all colonies were counted that conformed to the "enteric" designation (lactose fermentation with the production of green, metallic sheen). Organisms conforming to either the "enteric" or "coliform" designation were further differentiated by their ability to produce gas at 44.5 C in MR-VP medium (BBL) and by indole: methyl red: Voges-Proskauer: citrate (IMViC) classification (Geldreich, et al., 1966). The taxonomic classification of the isolates was confirmed by techniques suggested by Edwards and Ewing (1962) and the nomenclature used in the remainder of this study follows that proposed by Ewing (1963) which substitutes the generic name Enterobacter for Aerobacter. After these preliminary tests were completed, all organisms, including the stock cultures of Escherichia coli (ATCC 11775) and Enterobacter aerogenes (ATCC 12658) were maintained on Nutrient Agar (Difco) slants in the refrigerator for later use.

Gas production by Enterobacter: Each strain recovered was inoculated into a duplicate series of Durham fermentation tubes containing 1% concentrations of glucose (Difco), lactose (Difco), or sodium formate (Fisher Scientific Co., Pittsburgh, Pa.) to qualitate gas production. The basal medium was Nutrient Broth (Difco), and each tube was preincubated to equilibrate the medium to the desired experimental temperature. One set of fermentation tubes was incubated at 30.0 C and the other at 44.5 C, and all tubes were observed for collected gas in the inverted vials at 24 and 48 hr. After incubation, 1 ml of

20% KOH solution was added to each culture tube containing gas to absorb any evolved CO_2, and after 30 min of incubation at room temperature, the inverted vial was removed from the culture tube and the open end was placed in a flame to determine if H_2 was present.

Formic hydrogenlyase assay: Pure culture cell suspensions of the ATCC Escherichia and Enterobacter strains of aquatic enteric bacteria were prepared by inoculating each into flasks containing 200 ml of sterile, prewarmed Trypticase Soy Broth (BBL). These cultures were grown aerobically with vigorous shaking to avoid inducing the lyase system. Growth temperature was either 30.0 C or 44.5 C, depending on desired experiment, and cells were harvested after 18 hr of incubation. The cells were collected by centrifugation and washed three times in 0.1 M phosphate buffer, pH 6.8, and suspended in buffer to give an optical density (OD) of 0.2 at 540 nm with a Spectronic 20 (Bausch & Lomb, Inc., Rochester, N. Y.) colorimeter-spectrophotometer. Dry weights of cells were obtained by comparing the OD with a standard curve, which was prepared with organisms washed with deionized water and then dried overnight at 105 C.

Increased recovery rate of salmonellae from stream bottom sediments vs. surface waters: Much of our concern today for the presence of enteric pathogens in our surface waters is largely due to the increasing demands which are being placed upon this resource. Techniques have been developed which increase recovery rates of the occasional Salmonella or Shigella which can be found in high-quality surface water (Spino, 1966; Fair and Morrison, 1967) but the lack of their recovery does not preclude their presence at a particular sampling point.

Sampling procedures: In light of the increasing number of reports which suggest growth and multiplication of the coliform group of bacteria in natural waters (Kusnezow, 1959; Kitrell and Furfari, 1963; Hendricks, 1970) and that river and lake bottom sediments will stimulate their rate of growth (Boyd and Boyd, 1962; Hendricks and Morrison, 1967), it was speculated that recovery yields of pathogenic enteric bacteria might be higher than usually reported for surface waters if river bottom sediments were concomitantly sampled. For this study, several locations were selected on the North Oconee River (Clark County, Ga.) below an unchlorinated treated sewage outfall for periodic water and bottom sediment collection (Fig. 5). Samples were collected from each location by techniques suggested by the American Public Health Association (1966) and enriched for the Salmonella-Shigella group of organisms

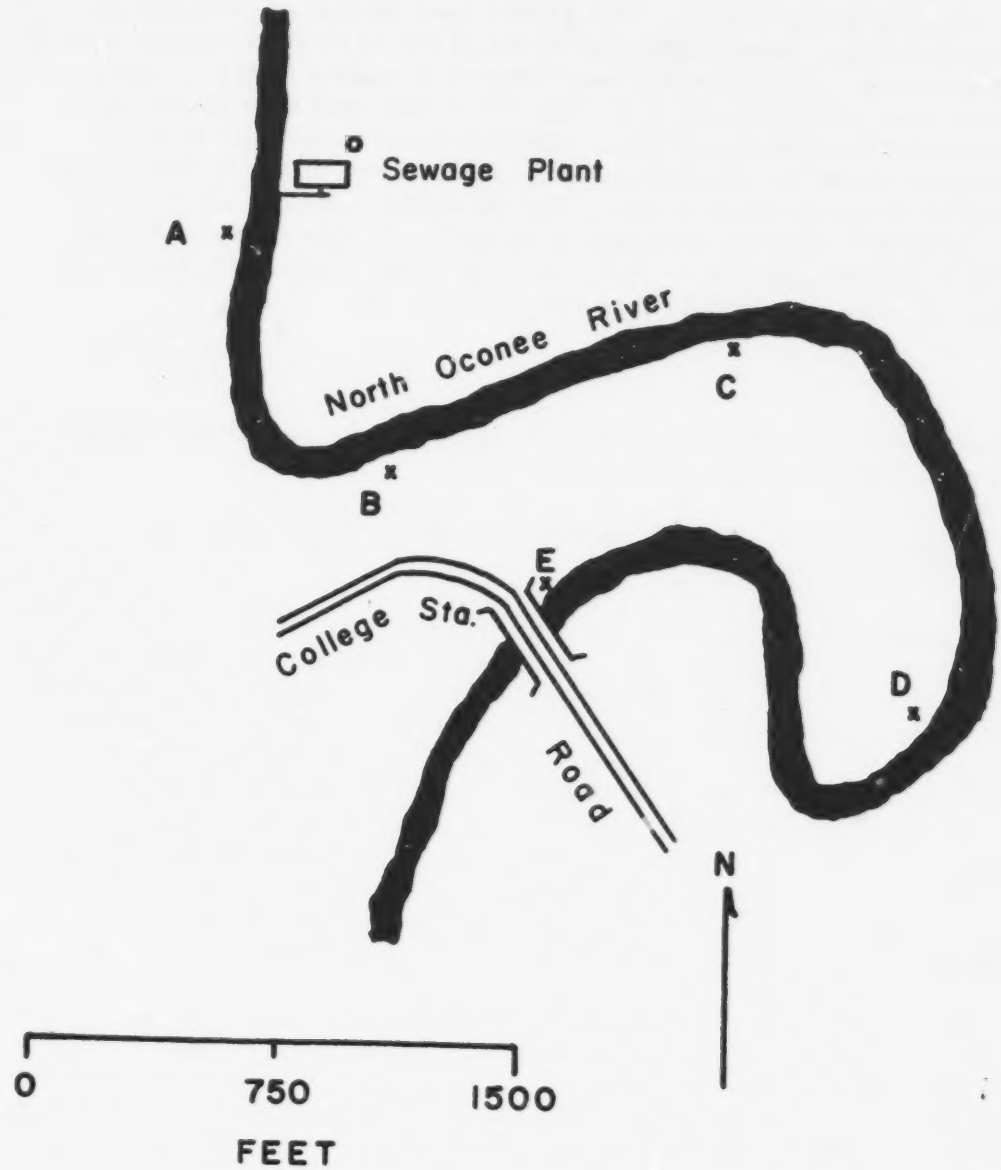


Fig. 5. Study locations below the Bailey St. sewage treatment plant for Salmonella recovery.

in Selenite Broth (Difco). Ten grams (wet weight) of sediment were inoculated into tubes containing 10 ml of the enrichment broth, whereas one liter of river water was filtered through a 0.45μ (HA) filter disc (Millipore Corp.). The filter disc was then placed into a similar Selenite Broth tube. After 18 hr of incubation at 37 C, samples of the enriched cultures were streaked to MacConkey Agar (Difco) for primary isolation. The taxonomic classification of the isolates was made by techniques suggested by Edwards and Ewing (1962) and was confirmed serologically by the Enteric Bacteriology Unit, Center for Disease Control, Atlanta, Ga.

Part 2

Evaluation of the Ability of Enteric Bacteria to Use Natural Aquatic Substrates by Oxygen Uptake Experiments

Enteric bacterial metabolism of substrates in river water and in stream sediment eluates

The concern today by environmentalists with the concentration of nutrients in freshwater lakes and streams is largely the result of our increasing demands which are being placed upon this resource. For some time it has been known that terrestrial bacterial species can grow and reproduce in extremely dilute nutrient concentrations (Butterfield, 1929; McGrew and Mallette, 1962; Hendricks and Morrison, 1967) of laboratory media, but most of these organisms are not involved in pathogenesis of man or higher animals. Enterobacteriaceae, however, not only contains bacteria which are indicators of fecal pollution, but others, such as Salmonella, Shigella, and Arizona, which can produce serious intestinal disease.

It is suspected that bottom sediments of lakes and streams play a major role in the recycling process of nutrients which allows for much of the observed heterotrophic growth (Harter, 1968), but the nature of the role is still quite vague. Studies by Malaney et al. (1962) and Boyd and Boyd (1962) indicate that sediments will stimulate the growth of bacterial species indigenous to freshwater lakes and streams. Work by Hendricks and Morrison (1967), though, has shown that stream sediments have the capacity to bind basal nutrients loosely and that aqueous extracts of sediments will increase the rate of growth of various enteric species in high-quality water at 15 C and less. It was postulated by these investigators that this loosely bound material was probably available for microbial use within the natural environment. The study is primarily concerned with nutrient binding by river bottom sediments and conditions for its removal and use by

enteric bacteria.

Study sites: Water and bottom sediments were collected for investigation from sites on the North Oconee River, a typical stream of the North Georgia piedmont in Clarke County, near Athens, Georgia. These sites are identical to those described earlier.

Organisms: One strain each of Escherichia coli, ATCC 11775; Enterobacter aerogenes (Aerobacter aerogenes), ATCC 12658; Proteus rettgeri, Arizona arizonae (Paracolobactrum arizonae), Shigella flexneri A1, NCDC (Atlanta, Georgia); and Salmonella senftenberg, CPHS (Ottawa, Canada), was grown in Trypticase Soy Broth (BBL) at 30 C for 16 hours. Cells were then harvested by centrifugation; washed 3 times in sterile, carbon-free, deionized water; incubated at 30 C for 4 hr to expend endogenous metabolism; and rested at 4 C for 18 hr before each experiment.

Experimental substrates: River water and stream bottom sediments were collected from each study site. The river water was immediately sterilized in an autoclave at 121 C for 15 min and then frozen after samples were removed for chemical analysis. After collection, sediments from each site were divided into 30 g lots, and each aliquot was washed three consecutive times with 50 ml volumes of carbon-free deionized water. These slurries were agitated for 30 min with a magnetic mixer and then clarified by centrifugation. Each washing was collected, sterilized in the autoclave, and frozen for later use.

After determining optimal pH and buffer ionic strength for elution, 30 g aliquots of washed sediment from each site were eluted with 50 ml of 0.3 M sodium phosphate buffer (Colowick and Kaplan, 1955), pH 7.0, in separate experiments, and with river water from the site where the sediment was collected. These eluates were also autoclaved at 121 C for 15 min and then frozen for later respiration studies and chemical analysis.

Analyses for nutritional constituents were made on the sediment washings and eluates as well as river water from each site by chemical procedures. Ammonia nitrogen and orthophosphate content was determined by procedures in Standard Methods (1966), hexose was measured by anthrone (Morris, 1948), and protein content was determined by the Folin-Ciocalteau procedure (Colowick and Kaplan, 1955).

Contamination of equipment by basal nutrients: In experiments of the nature that are reported here, it is extremely important that the dilute

nutrient substrates, including the river water, not be contaminated by extraneous basal nutrients. Major sources of possible contamination include the utensils, water, air, filters, and many other materials that may come in contact with the culture system.

In this investigation the major source of contamination was tap distilled water so it was necessary to employ a deionizing system that rendered the water carbon free as determined by CHN analyses on a one l. lyophilized water sample. All utensils used in this study were washed with detergent, well rinsed with tap water, and incubated overnight in a sulfuric acid-dichromate bath. The utensils were then rinsed with copious amounts of carbon free water, capped with paper and autoclaved. All membrane filters used to filter sterilized media were washed with hot carbon free water to remove soluble basal nutrients, and no culture device was employed with out suitable air filtration.

Bacterial respiration studies with eluted sediments: Cell suspensions of each rested bacterial culture were prepared in deionized water and standardized to 0.9 optical density at 540 nm with a Spectronic 20 (Bausch and Lomb, Rochester, N. Y.) colorimeter-spectrophotometer. Dry cell weights were obtained by comparing the optical density with a standard curve which was prepared with organisms washed with deionized water and then dried overnight at 105 C.

Respiration studies were carried out with the use of a Biological Oxygen Monitoring System (Cole-Parmer, Chicago, Ill.) in which 4 ml of a particular substrate were placed into the monitor with 2 ml of the standardized bacterial suspension, and oxygen uptake was measured for 15 min (Fig. 6). Individual experiments for each organism and substrate were run in duplicate at temperatures of 30 C, 20 C, and 5 C. Control substrates consisting of deionized water, river water from each site, varying dilutions of minimal salts-glucose medium (Davis, 1950), and minimal medium containing 0.1, 0.2, and 0.3 M phosphate were run in each temperature series. Temperature of incubation was controlled within ± 0.2 C with a Lauda-Brinkman K-2R Circulator (Westbury, N. Y.) and respiration was calculated as mg atoms O/hr. /mg dried cell weight (Pomeroy and Johannes, 1968) after correcting for endogenous activity.

Utilization of substrates in detritus samples and from slime capsules of Enterobacter aerogenes by the coliform bacteria Escherichia coli and Enterobacter aerogenes

Detritus: Detritus samples for respiration studies were recovered from the three study sites on the Oconee River by two different

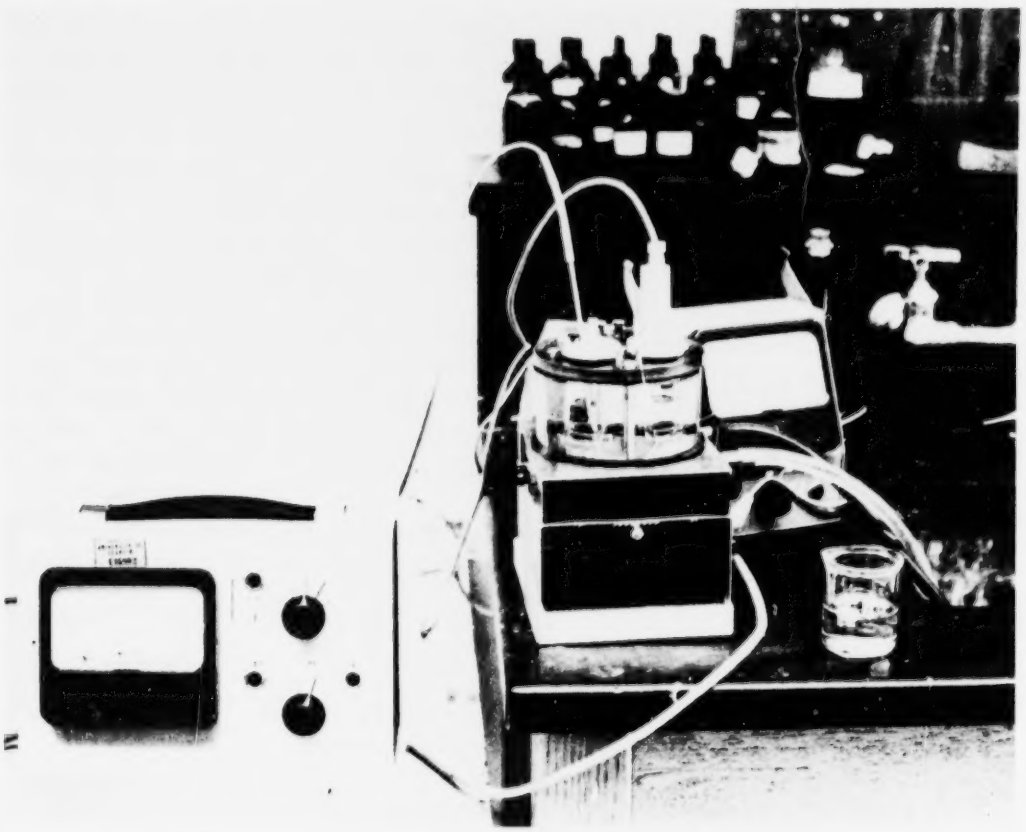


Fig. 6. Biological oxygen monitor (Yellow Springs Instruments, Inc.).

processes:

Aliquots of river water were dried at 105 C for 24 hrs and others lyophilized directly. The remaining solid material in each case was termed Suspended detritus and separately stored.

Particulate material present in river water was concentrated with the use of a device similar to the marine plankton concentrator of Pomeroy and Johannas (1968). This detritus was lyophilized and the remaining solid material was termed Concentrated detritus.

Slime: Polysaccharide capsular material (Slime) was produced from Enterobacter aerogenes by culturing the organism in a minimal salts-glucose medium (Davis, 1950). The slime that was produced in 18-24 hr was recovered by ethanol precipitation-cold centrifugation procedures (Colowick and Kaplan, 1955) and then lyophilized for later use.

Respiration studies: The detritus and slime samples were diluted with deionized water and used in respiration studies by techniques outlined above.

Part 3

Evaluation of the Ability of Enteric Bacteria to Use Natural Aquatic Substrates by Continuous Culture Experiments

It is generally assumed that basal nutrient concentrations present in most fresh water and marine systems are not sufficient for sustained bacterial growth. Although nitrogen and phosphorus are often in very short supply, growth of these microorganisms in water seems to be primarily limited by low concentrations of a suitable carbon and energy source. Studies of McGrew and Mallette (1962) have shown that approximately 5.0 μg/ml glucose is required just for maintenance of a bacterial culture, but this concentration is becoming more common in nature. These and other data by Herbert (1961) and by Tempest and Hunter (1965) suggest that a significant portion of the aquatic bacterial population may not be metabolically active, but it is also not inconceivable that a portion of the prototrophic bacterial population could be growing in situ at very low rates which cannot be easily detected.

To test this hypothesis in a fresh water system, the chemostat, a continuous culture apparatus has been employed which, when run at rate limiting substrate concentrations, allows for a growth rate equal to the rate at which the nutrient is diluted in the culture vessel.

Figure 7 is a schematic representation of the continuous culture system that was employed in this series of experiments while Figs. 8 and 9 consist of photographs of two different culture devices that were designed for these studies.

Preliminary experiments

The first experiments in this series were designed to answer four basic questions concerning the chemostat system and these included: 1. Of what significance is bacterial attachment to the glass walls of the chemostat? 2. Can very dilute minimal medium support the growth of a natural population of enteric bacteria from the Oconee River? 3. Can river water support the growth of natural populations of enteric bacteria? 4. At what dilution rates does bacterial growth take place?

Organisms: Bacteria present in an unsterilized aliquot of river water taken from below the sewage plant effluent (Site 3) served as an inoculum for these preliminary studies. 1.7 liters of the water was centrifuged to sediment the bacteria present, the supernatant was then poured off and the cells were then resuspended in an equal volume of the test substrate. These cells were then introduced into the chemostat to initiate the experiments.

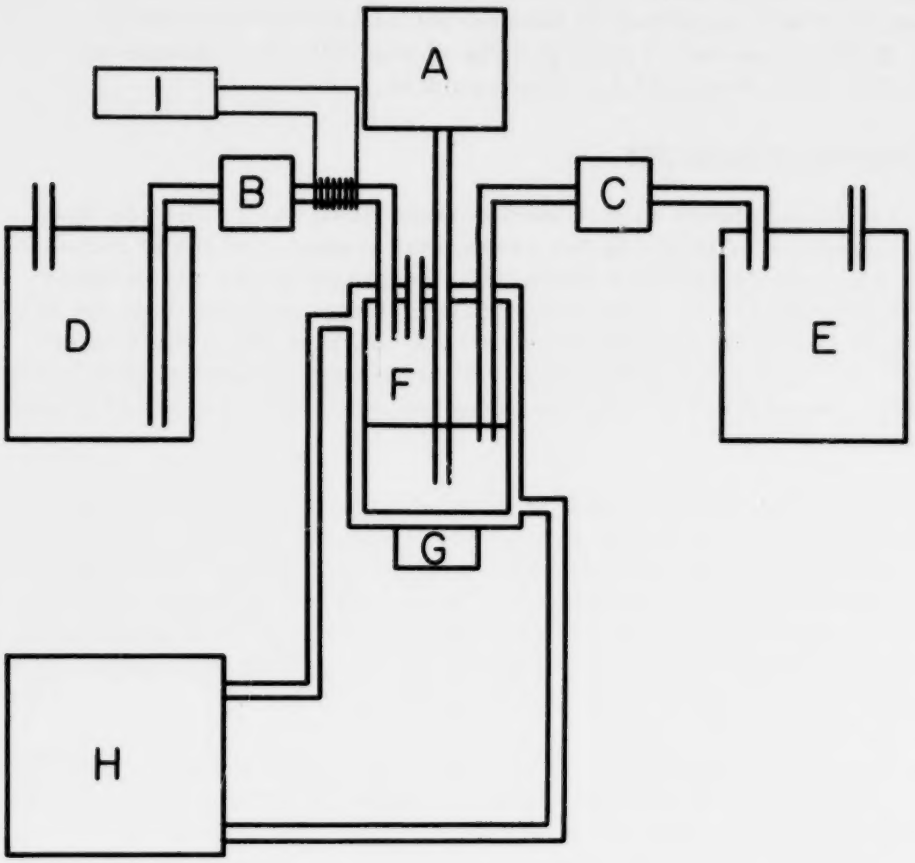
Experimental substrates: The two experimental substrates that were used in these studies included dilute minimal medium (Davis, 1950) and Oconee River water from site 3.

1. The minimal salts-glucose medium was prepared and sterilized and then diluted 1:1,000 with sterile, carbon free, deionized water. This medium was then introduced into the sterile chemostat for growth studies.
2. River water was collected by techniques mentioned previously and returned to the laboratory immediately for autoclaving (121 C for 20 minutes). After basal nutrient concentrations were determined, the water was ready for use as an experimental substrate.

Calculations: Herbert et al. (1956) has reported that during the transient state in a chemostat, a population (x) changes:

$$\frac{dx}{dt} = \mu x - Dx$$

or



A. Air Pump
B. Medium Pump
C. Waste Pump
D. Medium Reservoir
E. Waste Reservoir
F. Continuous Culture Apparatus
G. Magnetic Mixer
H. Circulator
I. Power Source and Coil

Fig. 7. Schematic design of the continuous culture system employed in this investigation.

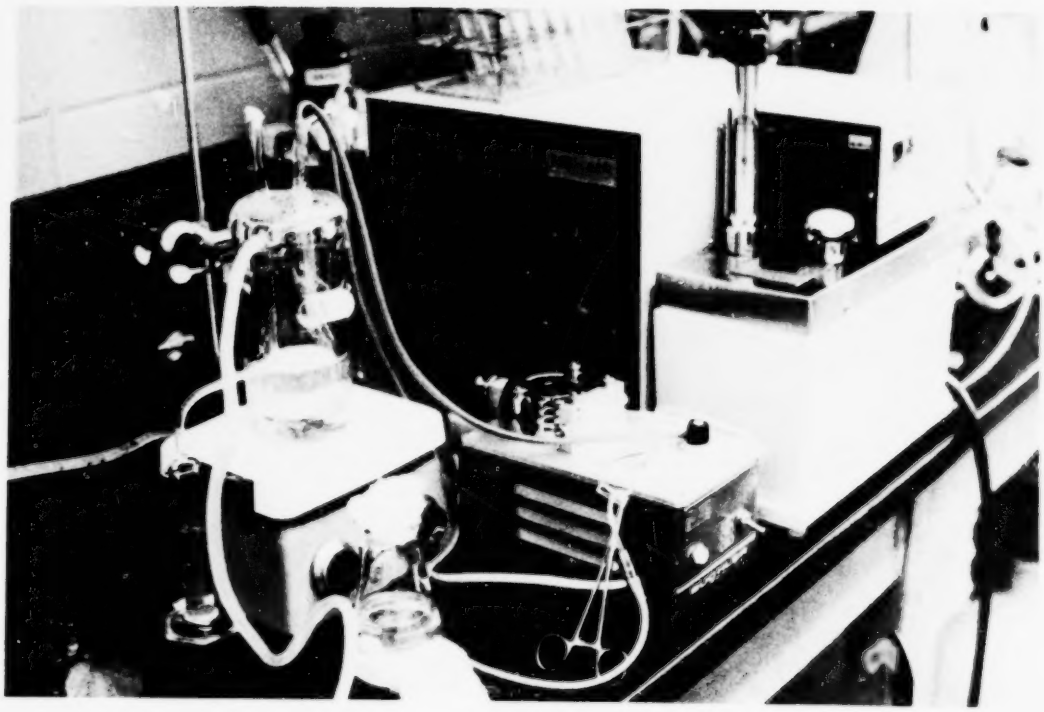


Fig. 8. 100 ml. continuous culture device.

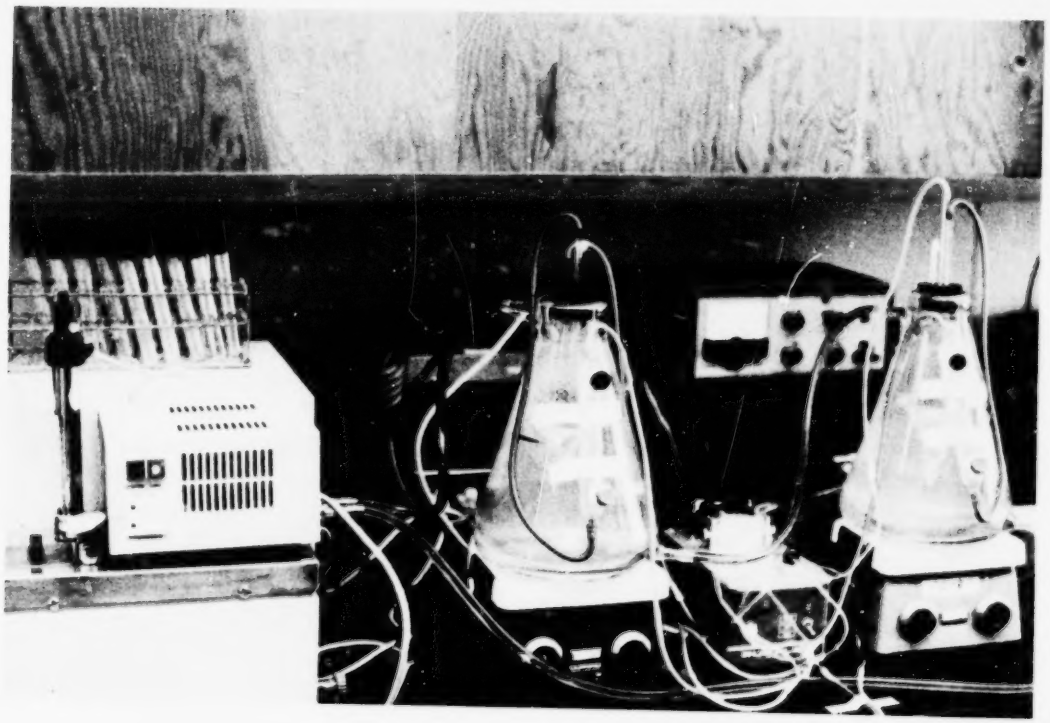


Fig. 9. 1.7 liter continuous culture device in duplicate.

$$x = x_o e^{(\mu - D)t}$$

Where x_o = initial population ($t = o$), μ = growth rate, and D = dilution rate.

In solving the previous equation for the growth rate (μ):

$$\mu = D + \frac{1}{t} \ln (x/x_o)$$

where

$$\frac{1}{t} \ln (x/x_o) = -A$$

and A equals the washout rate.

Therefore:

$$\mu = D - A \quad .$$

Jannasch (1969) has postulated four different cases of constant washout rates are conceivable in continuous culture studies:

1. The population doubles in one retention time and a steady state results.
2. The population grows at a rate greater than the washout rate.
3. The population does not grow and is washed out of the culture device.

4. The population disappears at a rate faster than the washout rate (death of the population).

These four cases are shown graphically in Fig. 10.

Bacterial attachment studies: In this series of experiments, the chemostat was run at 14 C and 26 C to more closely resemble in situ conditions. The dilution rate used at both experimental temperatures was 0.058 $hr.^{-1}$. In addition to determining suspended bacterial number and basal nutrient concentration, a series of glass microscope cover slips were placed into the chemostat for the enumeration of attached bacteria. Aliquots of water and a cover slip were removed at intervals over a 24 hour period. Counting procedures for the suspended bacteria were identical to those used previously, but the cover slips were first ground in a Waring blender with 100 ml sterile deionized water before aliquots were plated.

Dilute minimal medium and river water experiments: The chemostat experiments utilizing (Davis, 1950) Minimal Medium diluted 1:1,000 and river water from site 3 were run at an incubation temperature of 30 C and at a dilution rate of 0.012 $hr.^{-1}$. After the organisms had been introduced into the culture vessel, total enteric and coliform bacterial counts were determined daily on M-Standard Plate Count Broth (Difco) and M-Endo Broth (Difco) by the Millipore procedure, and duplicate plates were incubated at both 30 C and 44.5 C to estimate both nonfecal and fecal coliforms. Aliquots of water were also removed from the chemostats for analysis of basal nutrients when the counts were made.

Continuous culture experiments using river water and the collected detritus from the three sites as substrates

Data obtained in the preliminary experiments served as a basis to quantitate growth rates of six selected enteric bacterial species in river water and suspended and concentrated detritus samples from the three study locations on the North Oconee River.

Organisms: The enteric bacteria used in this portion of the investigation were prepared by the following procedure. One strain each of Escherichia coli, ATCC 11775; Enterobacter aerogenes (Aerobacter aerogenes) ATCC 12658; Proteus rettgeri, Arizona arizonae (Paracolobactrum arizonae), Shigella flexneri A1, NCDC (Atlanta, Georgia); and Salmonella senftenberg, CPHS (Ottawa, Canada), was grown in Trypticase Soy Broth (BBL) at 30 C for 16 hours. Cells were then harvested by centrifugation; washed 3 times in sterile, carbon-free,

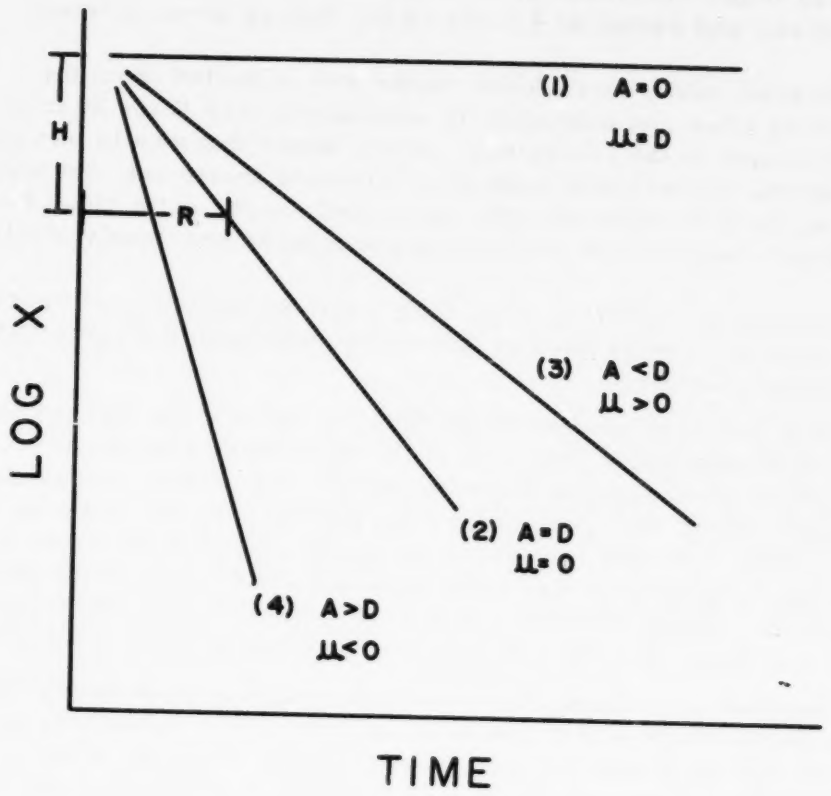


Fig. 10. Four possible cases of constant washout rates seen in continuous culture studies. A = washout rate, D = dilution rate, μ = growth rate, R = amount of time required to eliminate 1/2 (H) of the population. Jannasch, 1969.

deionized water; incubated at 30 C for 4 hr to expend endogenous metabolism; and rested at 4 C for 18 hr before an experiment.

Experimental substrates: River water was collected from the three study sites and immediately autoclaved (121 C for 20 min) upon returning to the laboratory. River water that was to be used as substrate immediately received no further treatment, but aliquots of the water from each site were processed for the collection of suspended and concentrated detritus by procedures previously mentioned.

Experimental procedures: The same basic procedures for operation of the chemostat were used in the studies with both the river water and detritus samples.

1. River water: In separate experiments, each of the washed and rested test organisms (usually 1 x 10^5 cells) were introduced into chemostats containing sterile water from each of the 3 sites, and each experiment was continued until a growth rate or a washout rate could be obtained with confidence at temperatures of 30 C, 20 C, and 5 C. The elapsed time was generally 200-450 hours for any particular experiment. Each chemostat was sampled daily for bacteriological counts and basal nutrients by procedures previously mentioned.

2. Suspended and concentrated detritus: Samples of suspended and concentrated detritus that had been collected over a two year period were pooled according to site, suspended in 5 l. of deionized water and sterilized in the autoclave. This material was then used as substrate for culture of E. coli at growth temperature of 30, 20, and 5 C. Each experiment was allowed to continue until either a growth or a washout rate could be calculated.

Parasitic activity of Bdellovibrio bacteriovorus against E. coli in river water

Even though enteric bacteria are capable of growth in fresh water systems, their numbers certainly do not increase continually without limit. In fact, their numbers decrease dramatically down stream from sewage outfalls. Interest in the bacterial parasite Bdellovibrio bacteriovorus initially began through reports of this organism attacking Gram negative bacteria and primarily cells of E. coli (Stolp and Starr, 1965; Varon and Shilo, 1969; Gillis and Nakamura, 1970). It was conceivable to us that such an organism could be involved in various mechanisms of biological stream self-purification if the organism could be maintained in the fresh water environment and a study was begun to evaluate its response to E. coli in the chemostat.

Organisms: The strain of B. bacteriovorus used in this study was recovered from bottom sediments at site 2, and the organism is an obligate parasite for E. coli , ATCC 11775.

The technique for isolating the B. bacteriovorus strain was essentially that of Stolp and Starr (1963) and consisted of suspending 200 g of sediment (wet wt) in 200 ml of deionized water and shaking vigorously for 1 hour. The suspension was centrifuged for 5 minutes at 200 rpm, and then the supernatant (containing all types of microbes extracted from the soil) was submitted to a series of differential filtrations. The stepwise filtration through Millipore filters of different porosities was started with filters of 3 μ average pore size diameter, and continued with filters of 1.2, 0.8, 0.65 and 0.45 μ pore size. Portions of the last 2 or 3 fractions, which have a diminishing content or ordinary bacteria, were then mixed with a suspension of the host strain. These mixtures were then plated in the same way that is done in phage isolation experiments by using the double-layer technique. If the added fraction of the soil filtrate contains the parasitic organisms, B. bacteriovorus lytic spots appear after 2-4 days which, in their initial stages, are externally identical to phage plaques. The isolation of Bdellovibrio from sewage or other material can be undertaken in a fashion similar to that described here.

Culture media: The technique for culturing the parasite is very much like that used to culture bacteriophages. The basic yeast peptone (YP) medium consists of a solid base layer and a semi-solid top layer. The media used are as follows:

1. Base layer medium: 1000 ml deionized water; 3 g Difco yeast extract; 0.6 g Difco peptone; 15 g agar; pH 7.2.
2. Top layer medium: 1000 ml deionized water; 3 g Difco yeast extract; 0.6 g Difco peptone; 6 g agar; pH 7.2.
3. YP Solution: 1000 ml deionized water; 3 g Difco yeast extract; 0.6 g Difco Peptone; pH 7.2.

After the bottom layer solidified a top layer was overlaid with 0.5 ml portions of a washed E. coli cell suspension and of the filtrate which is to be checked for Bdellovibrio mixed with about 4 ml of the molten semi-solid agar YP medium. After overnight growth, the plates are checked for phage plaques. Although the phage content of river water or sewage samples is usually extremely low, phage plaques occur occasionally and must be marked at this point in order to avoid confusion with the parasite plaques which develop somewhat more slowly. Bdellovibrio normally requires at least 2 days in order to develop visible plaques in isolation experiments since the single parasitic cells

embedded in the lawn of growing bacteria have to overcome their own lag phase before they can start multiplying. Plaques in isolation plates, which are suspected to be caused by Bdellovibrio, were cut out from the top layer, suspended in YP solution, and plated in a lawn of the host bacteria by using a dilution series to get single plaque formation. If lytic spots developed after 2 days of incubation, some of the plaques were checked microscopically for the presence of parasites.

Purification: Parasites from a single plaque were suspened in sterile YP solution, which was then passed through a 0.45 μ Millipore filter, diluted, and plated for plaque formation with an excess of the homologous host. After 3 successive single plaque isolations, the strain was regarded as pure in the sense of representing the descendants of a single cell (i.e., a clone). The organisms recovered were maintained in a YP medium culture of E. coli at 5 C until use.

B. bacteriovorus activity in river water: After preliminary experiments to compare the growth of B. bacteriovorus in a nutritionally rich YP medium with that achieved in river water on E. coli, the parasite was introduced into chemostat containing an actively growing culture of E. coli at 30 C. Counts of both organisms were determined daily by techniques outlined above, and basal nutrient concentrations were also monitored.

SECTION V

RESULTS

The following results were obtained during the investigation of Enteric Bacterial Degradation of Stream Detritus which was active from August, 1968 through July, 1971. These results will be presented as follows:

1. Water quality analysis of the North Oconee River at three selected sites at Athens, Georgia.
2. Evaluation of the ability of enteric bacteria to use natural aquatic substrates by oxygen uptake experiments.
3. Evaluation of the ability of enteric bacteria to use natural aquatic substrates by continuous culture experiments.

Part 1

Water Quality Analysis of the North Oconee River at three selected sites at Athens, Georgia

Routine analyses

Chemical and bacterial assays: Tables 1 through 18 contain data obtained from chemical and bacteriological analyses of the North Oconee River water at three study sites near Athens, Georgia. These data are presented in the form of monthly means of at least two samples. Concentrations of basal nutrients are expressed in terms of mg/l while bacteriological counts are shown as the count/ml of river water. In each case, the means have been rounded off to the first experimental significant digit. Table 19 contains results from the Carbon-Hydrogen-Nitrogen analyses of the dried dissolved solids from each of the study sites over the complete study period.

Procedure evaluations

Formic hydrogenlyase induction as a basis for the Eijkman fecal coliform concept: A total of 629 isolates were recovered from the North Oconee River (Site 3) over a 1-year period (1968-1969) and were broadly categorized into either the "coliform" or "enteric" groups on the basis of their cultural reactions on Endo medium. These organisms were further differentiated into Escherichia, Enterobacter, and intermediate groups by the IMViC procedure and for gas production in glucose at 44.5 C (Table 20). Geldreich (1964) reported that enteric bacterial stains producing gas at the elevated temperature

Table 1. Mean analysis of nutritional factors in the North Oconee River at Site 1 during 1968-1969

Test	Month												Mean 68-69
	1968 Aug	Sept	Oct	Nov	Dec	1969 Jan	Feb	Mar	Apr	May	June	July	
Temp(°C)	23.5	19.5	17.0	9.0	4.5	8.0	7.0	10.5	16.5	17.0	23.5	24.5	15.0
pH	7.0	6.9	6.9	6.8	6.9	7.0	6.9	7.0	7.2	6.1	7.2	7.0	6.9
DO(%Sat.)	64.0	56.5	65.0	93.0	87.0	59.0	48.0	47.0	80.0	63.0	85.0	75.5	68.5
BOD	2.1[a]	2.3	2.2	2.2	2.2	1.0	2.0	2.1	1.8	2.1	2.4	3.2	2.1
Nitrogen[b]	- [c]	-	-	2.0	0.5	3.9	0.5	0.5	0.5	0.5	4.3	1.6	3.2
Hexose	2.5	1.8	1.4	3.0	1.6	0.8	1.8	1.1	1.0	1.0	2.1	0.8	1.6
Phosphate	2.0	2.0	0.8	1.7	0.9	3.6	0.2	0.4	0.6	1.2	0.6	0.2	1.2
Protein	10.0	11.5	14.0	18.0	12.5	21.7	5.0	3.0	2.0	20.0	6.0	1.0	10.4
Solids[d]	77.0	105.0	60.0	126.0	56.0	56.0	447.0	127.0	54.0	591.0	205.0	45.0	158.0

[a] Concentration expressed in Mg/liter.

[b] Ammonia Nitrogen.

[c] Data not collected.

[d] Dissolved solids.

Table 2. Mean analysis of nutritional factors in the North Oconee River at Site 2 during 1968-1969

Test	Month												Mean 68-69
	1968 Aug	Sept	Oct	Nov	Dec	1969 Jan	Feb	Mar	Apr	May	June	July	
Temp(^{o}C)	24.0	20.0	17.0	9.5	4.5	7.5	7.0	10.0	17.0	17.0	24.0	24.5	15.2
pH	6.9	7.0	7.0	6.6	7.0	7.0	6.9	7.0	7.4	7.0	7.0	6.9	7.0
DO(%Sat.)	69.0	59.0	46.0	53.0	57.0	35.0	46.0	50.0	52.0	53.0	98.0	80.0	58.2
BOD	2.1[a]	2.3	2.2	2.2	1.9	1.5	2.3	1.7	1.6	1.2	2.4	2.4	2.0
Nitrogen[b]	-[c]	-	-	2.5	0.5	2.9	0.5	0.5	1.2	7.5	3.1	2.1	2.3
Hexose	2.5	2.5	3.2	3.5	2.5	1.2	1.8	1.0	1.5	5.5	2.5	2.0	2.5
Phosphate	2.8	1.2	0.6	1.9	1.0	3.6	4.0	1.8	1.6	2.1	0.7	0.4	1.8
Protein	10.0	15.5	16.2	17.5	9.7	21.7	3.0	7.5	5.0	25.0	7.0	2.2	11.7
Solids[d]	120.0	84.0	62.0	88.0	51.0	69.0	423.0	180.0	72.0	202.0	185.0	94.0	134.0

[a] Concentration expressed in Mg/liter.

[b] Ammonia nitrogen.

[c] Data not collected.

[d] Dissolved solids.

Table 3. Mean analysis of nutritional factors in the North Oconee River at Site 3 during 1968-1969

Test	Month												Mean 68-69
	1968 Aug	Sept	Oct	Nov	Dec	1969 Jan	Feb	Mar	Apr	May	June	July	
Temp(°C)	24.5	20.5	17.5	9.0	5.0	8.0	8.0	11.0	17.0	17.0	23.5	24.5	15.5
pH	6.9	7.0	7.0	6.4	6.3	7.1	6.9	7.3	7.0	7.4	7.2	7.0	7.0
DO(%Sat.)	60.0	48.0	35.0	54.0	47.0	30.0	33.0	36.0	39.0	43.0	74.5	47.5	45.6
BOD	1.8[a]	3.0	3.6	3.5	3.9	3.8	2.8	3.1	2.5	2.9	5.7	3.7	3.4
Nitrogen[b]	-[c]	-	-	7.0	3.2	7.2	1.9	2.2	4.4	6.4	5.1	4.8	4.7
Hexose	3.0	1.0	1.8	2.2	2.1	1.3	1.1	1.0	2.0	3.0	1.0	0.6	1.7
Phosphate	1.9	1.6	2.8	2.7	3.3	9.4	0.8	3.2	2.0	15.2	0.5	0.7	3.7
Protein	10.0	15.0	18.5	27.0	17.0	22.3	5.5	11.8	7.0	25.0	11.0	3.5	14.5
Solids[d]	107.0	144.0	91.5	126.5	74.0	94.0	500.0	223.0	66.0	152.0	83.0	107.0	147.0

[a] Concentration expressed in Mg/liter.

[b] Ammonia Nitrogen.

[c] Data not collected.

[d] Dissolved solids.

Table 4. Mean bacteriological counts[a] from the North Oconee River at Site 1 during 1968-1969

	Heterotrophic Bacteria		Enteric Bacteria			
	30 °C	44.5 °C	Total 30 °C	Coliforms 30 °C	Total 44.5 °C	Coliforms 44.5 °C
Aug 68	10,000	-[b]	1,300	200	-	-
Sept	9,700	116	290	100	283	126
Oct	1,800	149	1,700	315	61	34
Nov	2,000	87	3,200	400	40	15
Dec	370	66	19	0	16	6
Jan	7,400	200	5,200	2,820	3	3
Feb	730	60	1	0	4	3
Mar	430	68	40	25	0	0
Apr	150	30	30	20	1	1
May	1,300	10	1,200	0	0	0
June	320	15	28	1	2	2
July	2,500	545	40(	90	100	0
Mean 68-69[c]	3,100	122	1,200	330	46	17

[a] Counts/ml of river water.

[b] Data not collected.

[c] Means have been rounded off to the first significant digit.

Table 5. Mean bacteriological counts[a] from the North Oconee River at Site 2 during 1968-1969

	Heterotrophic Bacteria		Enteric Bacteria			
	30 °C	44.5 °C	Total 30 °C	Coliform 30 °C	Total 44.5 °C	Coliform 44.5 °C
Aug 68	40,000	-[b]	1,500	300	-	-
Sept	3,400	590	450	230	100	44
Oct	2,900	730	1,700	625	650	460
Nov	40,000	1,200	18,000	10,000	950	210
Dec	1,800	447	625	470	98	43
Jan 69	5,400	197	1,900	800	1	1
Feb	140	33	40	10	2	1
Mar	300	14	60	40	0	0
Apr	430	32	350	100	7	7
May	1,200	100	1,400	0	4	0
June	380	52	19	3	55	18
July	2,000	120	750	80	30	0
Mean 68-69[c]	8,200	320	2,200	1,100	170	70

[a] Count/ml river water.

[b] Data not collected.

[c] Means have been rounded off to the first significant digit.

Table 6. Mean bacteriological counts[a] from the North Oconee River at Site 3 during 1968-1969

	Heterotrophic Bacteria		Enteric Bacteria			
	30 °C	44.5 °C	Total 30 °C	Coliform 30 °C	Total 44.5 °C	Coliform 44.5 °C
Aug 68	200,000	-[b]	10,000	1,000	-	-
Sept	35,000	27,000	7,800	1,900	1,500	290
Oct	280,000	2,000	3,300	610	1,400	800
Nov	150,000	5,400	15,000	4,400	630	321
Dec	12,000	590	12,000	1,200	227	92
Jan 69	13,000	360	11,000	1,100	50	36
Feb	3,400	610	2,800	975	331	260
Mar	5,600	170	4,400	3,800	1	0
Apr	7,800	300	1,400	1,100	80	72
May	150,000	350	2,800	63	10	0
June	30,000	390	2,500	280	38	31
July	80,000	1,300	1,800	300	1,200	430
Mean 68-69[c]	74,000	3,500	6,200	1,900	450	210

[a] Count/ml river water.

[b] Data not collected.

[c] Means have been rounded off to the first significant digit.

Table 7. Mean analysis of nutritional factors in the North Oconee River at site 1 during 1969-1970

Test	Month												Mean 69-70
	1969 Aug	Sept	Oct	Nov	Dec	1970 Jan	Feb	Mar	Apr	May	June	July	
Temp(°C)	22.5	22.0	14.0	9.0	5.0	0.5	6.5	12.5	17.5	17.0	23.5	22.5	14.4
pH	7.0	6.9	6.8	7.0	6.7	7.1	7.0	6.9	7.2	7.0	7.3	6.7	7.0
DO(%Sat.)	98.0	107.0	93.0	95.5	83.0	92.5	102.0	90.0	78.0	90.0	75.0	78.5	90.2
BOD	6.8[a]	3.1	3.0	1.5	1.5	3.4	2.7	2.4	1.5	2.3	3.5	0.8	2.7
Nitrogen[b]	0.5	0.5	0.1	0.2	0.5	0.4	0.6	0.6	0.6	0.8	0.9	0.4	0.5
Hexose	1.0	1.2	0.8	0.5	0.0	0.5	0.8	0.8	0.6	0.2	5.5	0.1	1.0
Phosphate	0.7	0.6	0.4	0.2	0.2	0.4	0.4	0.4	0.6	0.5	0.8	0.5	0.4
Protein	2.0	1.5	1.4	1.2	1.9	1.5	7.1	2.0	2.8	1.9	2.2	4.9	2.5
Solids[c]	69.0	60.0	164.0	183.0	354.0	60.0	95.0	63.0	75.0	50.0	38.0	91.5	108.5

[a] Concentration expressed in Mg/liter.

[b] Ammonia nitrogen.

[c] Dissolved solids.

Table 8. Mean analysis of nutritional factors in the North Oconee River at Site 2 during 1969-1970

Test	Month												Mean 69-70
	1969 Aug	Sept	Oct	Nov	Dec	1970 Jan	Feb	Mar	Apr	May	June	July	
Temp(°C)	23.0	22.5	15.0	10.5	6.0	1.5	8.0	12.5	18.0	17.0	24.0	23.2	15.1
pH	6.7	6.8	6.5	6.7	6.6	6.9	7.0	6.8	7.2	7.0	7.2	6.8	6.8
DO(%Sat.)	67.0	92.0	100.0	102.0	82.5	94.0	102.5	88.0	90.5	96.0	82.0	80.5	89.8
BOD	4.5[a]	1.7	2.0	7.0	4.9	3.6	2.0	1.8	2.0	1.4	1.0	2.4	2.8
Nitrogen[b]	0.8	0.6	0.2	0.3	0.9	0.4	0.7	0.6	0.6	0.8	0.8	0.8	0.6
Hexose	1.8	1.7	0.8	1.1	0.7	1.0	0.4	1.0	0.7	0.2	0.5	0.0	0.8
Phosphate	0.8	0.6	0.7	0.1	0.7	0.3	0.4	0.6	0.5	0.6	0.6	0.5	0.5
Protein	4.0	1.5	1.4	1.2	3.3	2.2	6.6	2.8	2.7	3.4	1.2	1.4	2.6
Solids[c]	93.0	84.0	165.0	172.0	364.0	61.0	58.0	69.0	78.0	60.0	30.0	78.0	109.3

[a] Concentration expressed in Mg/liter.

[b] Ammonia nitrogen.

[c] Dissolved solids.

Table 9. Mean analysis of nutritional factors in the North Oconee River at Site 3 during 1969-1970

Test	Month												Mean 69-70
	1969 Aug	Sept	Oct	Nov	Dec	1970 Jan	Feb	Mar	Apr	May	June	July	
Temp(°C)	23.0	22.5	15.5	10.5	6.0	1.5	7.5	13.0	18.0	17.0	23.5	23.2	15.1
pH	6.8	6.9	6.6	6.7	6.6	6.8	6.8	6.8	6.2	7.0	7.0	6.8	6.7
DO(%Sat.)	81.5	67.5	87.5	92.5	83.0	80.5	90.0	90.0	69.0	86.5	57.0	50.0	71.2
BOD	6.8[a]	4.2	7.6	4.5	3.4	5.0	4.6	2.9	1.9	2.2	4.7	4.1	4.3
Nitrogen[b]	1.8	2.0	1.1	1.0	1.9	1.2	9.2	1.1	1.6	1.2	2.1	2.8	2.3
Hexose	1.7	2.1	1.2	0.9	0.8	0.8	1.4	0.8	1.1	1.7	1.1	0.6	1.1
Phosphate	1.0	1.6	1.2	0.4	1.9	0.9	1.0	0.8	1.1	0.9	1.3	2.0	1.2
Protein	4.0	6.5	3.2	2.4	9.9	5.1	6.9	3.2	4.7	4.3	4.5	5.8	5.0
Solids[c]	82.0	91.0	187.0	160.0	385.0	95.0	70.0	71.0	78.0	63.0	31.0	86.5	116.6

[a] Concentration expressed in Mg/liter.

[b] Ammonia nitrogen.

[c] Dissolved solids.

Table 10. Mean bacteriological counts[a] from the North Oconee River at Site 1 during 1969-1970

	Heterotrophic Bacteria		Enteric Bacteria			
	30 °C	44.5 °C	Total 30 °C	Coliform 30 °C	Total 44.5 °C	Coliform 44.5 °C
Aug 69	3,100	100	350	0	35	0
Sept	1,300	125	700	200	205	55
Oct	775	190	265	0	55	14
Nov	515	80	130	15	5	5
Dec.	1,100	160	154	60	0	0
Jan 70	2,800	10	330	30	5	5
Feb	2,000	780	115	25	0	0
Mar	575	110	200	30	0	0
Apr	375	10	210	30	0	0
May	880	240	650	60	100	20
June	1,200	260	200	0	40	10
July	10,000	3,000	8,800	2,800	2,000	200
Mean 69-70[b]	2,100	420	1,000	270	200	25

[a] Counts/ml river water.

[b] Means have been rounded off to the first significant digit.

Table 11. Mean Bacteriological counts [a] from the North Oconee River at Site 2 during 1969-1970

	Heterotrophic Bacteria		Enteric Bacteria			
	30 °C	44.5 °C	Total 30 °C	Coliform 30 °C	Total 44.5 °C	Coliform 44.5 °C
Aug 69	3,900	250	1,700	100	375	110
Sept	12,000	160	1,600	100	325	105
Oct	2,700	280	800	150	310	140
Nov	11,000	1,800	570	170	750	61
Dec	5,000	380	940	700	70	45
Jan 70	2,400	20	320	100	10	5
Feb	1,800	145	135	30	0	0
Mar	995	105	245	55	0	0
Apr	535	55	270	25	0	0
May	2,100	160	880	60	150	0
June	1,700	500	400	400	40	10
July	6,000	3,600	10,000	3,300	3,000	200
Mean 69-70 [b]	4,100	620	1,500	430	420	56

[a] Count/ml river water.

[b] Means have been rounded off to the first significant digit.

Table 12. Mean bacteriological counts[a] from the North Oconee River at Site 3 during 1969-1970

	Heterotrophic Bacteria		Enteric Bacteria			
	30 °C	44.5 °C	Total 30 °C	Coliform 30 °C	Total 44.5 °C	Coliform 44.5 °C
Aug 69	10,000	300	8,000	100	430	170
Sept	250,000	18,000	30,000	4,600	1,600	1,100
Oct	7,500	4,500	955	500	900	500
Nov	3,400	4,000	1,500	700	1,200	730
Dec	5,100	3,400	5,600	2,000	700	400
Jan 70	5,100	200	3,500	1,800	105	100
Feb	10,000	725	5,700	2,200	80	60
Mar	2,100	330	780	370	25	15
Apr	91,000	245	4,900	3,200	50	30
May	3,000	200	980	190	900	300
June	4,700	1,900	4,900	1,100	5,000	1,100
July	800,000	34,000	240,000	37,000	13,000	7,600
Mean 69-70[b]	100,000	5,700	26,000	4,500	2,000	1,000

[a] Count/ml of river water.

[b] Means have been rounded off to the first significant digit.

Table 13. Mean analysis of nutritional factors in the North Oconee River at Site 1 during 1970-1971

Test	Month												Mean
	1970 Aug	Sept	Oct	Nov	Dec	1971 Jan	Feb	Mar	Apr	May	June	July	70-71
Temp(°C)	24.5	24.0	15.0	9.5	7.0	2.5	6.0	5.0	15.0	17.5	23.0	22.5	14.5
pH	6.8	6.7	6.8	6.9	7.0	6.8	6.7	7.8	7.3	7.6	7.2	7.4	7.1
DO(%Sat.)	92.0	83.0	119.0	100.0	81.5	95.0	88.0	93.0	79.0	79.5	82.5	74.5	88.9
BOD	1.0[a]	1.5	2.0	3.8	1.2	2.2	1.1	1.5	1.0	0.8	0.7	0.9	1.5
Nitrogen[b]	0.6	0.9	0.4	0.1	0.7	0.3	0.3	0.6	1.1	1.1	0.8	0.9	0.6
Hexose	1.0	1.0	0.3	2.7	-[c]	0.5	0.4	2.4	7.0	4.3	3.4	4.7	2.5
Phosphate	0.7	0.3	1.2	0.5	0.4	0.2	0.3	0.6	1.2	2.0	2.2	2.8	1.0
Protein	1.5	2.9	1.1	1.2	2.3	2.0	1.2	3.2	4.9	4.5	3.8	6.2	2.9
Solids[d]	73.5	112.5	83.0	58.0	95.0	37.5	78.0	104.0	35.0	94.5	90.0	143.5	83.7

[a] Concentration expressed in Mg/liter.

[b] Ammonia Nitrogen.

[c] Data not collected.

[d] Dissolved solids.

Table 14. Mean analysis of nutritional factors in the North Oconee River at Site 2 during 1970-1971

Test	Month												Mean
	1970 Aug	Sept	Oct	Nov	Dec	1971 Jan	Feb	Mar	Apr	May	June	July	70-71
Temp(°C)	25.0	24.0	15.5	9.5	7.0	3.0	6.5	6.0	15.5	18.5	24.0	23.5	15.0
pH	6.6	6.7	6.8	6.8	7.0	6.9	6.4	7.3	6.9	7.1	7.4	6.9	6.9
DO(%Sat.)	83.0	76.0	113.5	101.5	87.5	96.5	90.0	97.0	86.5	85.5	86.0	82.0	90.4
BOD	0.6[a]	1.2	2.0	3.3	1.9	1.9	2.6	2.8	0.8	1.4	0.6	2.4	1.8
Nitrogen[b]	0.8	1.0	0.6	0.6	0.9	0.4	0.4	0.7	1.1	1.0	0.7	1.0	0.8
Hexose	1.4	1.4	0.4	2.2	-[c]	0.2	1.4	1.9	6.0	5.0	2.4	4.2	2.4
Phosphate	0.9	0.5	1.9	2.8	0.4	0.2	0.3	0.8	1.3	1.6	1.8	4.4	1.4
Protein	1.5	3.2	1.6	1.9	4.2	1.8	1.3	3.7	5.2	3.5	3.0	9.8	3.4
Solids[d]	73.5	125.5	88.0	74.0	92.0	49.0	53.5	105.0	39.0	79.5	91.0	179.5	84.2

[a] Concentration expressed in Mg/liter.

[b] Ammonia Nitrogen.

[c] Data not collected.

[d] Dissolved solids.

Table 15. Mean analysis of nutritional factors in the North Oconee River at Site 3 during 1970-1971

Test	Month												Mean
	1970 Aug	Sept	Oct	Nov	Dec	1971 Jan	Feb	Mar	Apr	May	June	July	70-71
Temp(°C)	25.0	25.0	16.0	9.5	7.0	3.0	6.5	6.0	15.0	18.5	24.0	23.5	15.0
pH	6.9	6.8	7.0	7.1	6.9	6.9	6.4	7.0	7.2	7.2	7.0	6.7	6.9
DO(%Sat.)	66.0	75.0	127.0	96.0	83.0	90.0	88.5	90.5	83.5	77.5	71.5	74.0	85.2
BOD	4.0[a]	3.0	3.6	4.9	3.6	1.3	2.8	2.9	2.3	3.5	2.7	3.1	3.1
Nitrogen[b]	2.2	1.4	1.2	1.0	1.0	0.9	0.8	1.1	1.4	1.4	1.4	1.4	1.3
Hexose	1.5	1.7	1.2	2.0	2.0	1.3	0.7	1.7	8.2	4.3	3.0	5.7	2.8
Phosphate	1.9	1.0	4.5	2.1	0.8	0.4	0.5	1.0	1.3	2.0	3.4	5.6	2.0
Protein	4.6	5.5	1.4	1.9	2.9	3.8	4.8	5.1	6.5	5.6	6.3	11.3	5.0
Solids[c]	74.0	129.5	121.0	77.0	90.0	51.0	70.0	115.0	54.5	89.5	92.0	212.0	98.0

[a] Concentration expressed in Mg/liter.

[b] Ammonium nitrogen.

[c] Dissolved solids.

Table 16. Mean bacteriological counts[a] from the North Oconee River at Site 1 during 1970-1971

	Heterotrophic Bacteria		Enteric Bacteria			
	30 °C	44.5 °C	Total 30 °C	Coliform 30 °C	Total 44.5°C	Coliform 44.5 °C
Aug 70	2,600	95	470	220	10	5
Sept	14,000	5,700	3,000	1,100	550	530
Oct	9,000	500	1,000	200	10	8
Nov	18,000	30	15,000	20	2	0
Dec	5,000	100	2,800	320	20	0
Jan 71	572	144	288	29	11	1
Feb	1,200	35	112	12	2	1
Mar	2,200	152	279	74	7	4
Apr	1,500	525	966	32	10	1
May	30,000	170	1,100	85	13	3
June	1,400	75	595	40	7	1
July	15,000	1,600	5,500	930	470	330
Mean 70-71[b]	8,400	757	2,600	255	102	73

[a]Count/ml river water.

[b]Means have been rounded off to the first significant digit.

Table 17. Mean bacteriological counts[a] from the North Oconee River at Site 2 during 1970-1971

	Heterotrophic Bacteria		Enteric Bacteria			
	30 °C	44.5 °C	Total 30 °C	Coliform 30 °C	Total 44.5 °C	Coliform 44.5 °C
Aug 70	2,800	695	1,300	200	155	100
Sept	55,000	2,100	18,000	5,000	750	250
Oct	24,000	1,000	3,500	900	80	10
Nov	15,000	1,400	2,100	300	70	10
Dec	16,000	410	5,900	700	600	100
Jan 71	6,600	971	292	51	22	4
Feb	1,800	86	209	44	5	3
Mar	2,400	63	480	107	8	5
Apr	6,600	150	2,100	270	24	8
May	2,100	148	1,300	85	16	3
June	3,000	234	525	55	22	3
July	18,000	930	6,200	1,200	225	131
Mean 70-71[b]	13,000	682	3,500	746	164	52

[a]Count/ml river water.

[b]Means have been rounded off to the first significant digit.

Table 18. Mean bacteriological counts[a] from the North Oconee River at Site 3 during 1970-1971

	Heterotrophic Bacteria		Enteric Bacteria			
	30 °C	44.5 °C	Total 30 °C	Coliform 30 °C	Total 44.5 °C	Coliform 44.5 °C
Aug 70	450,000	26,000	190,000	60,000	7,700	3,600
Sept	100,000	9,500	76,000	3,000	5,000	1,500
Oct	120,000	60,000	24,000	13,000	1,500	300
Nov	85,000	3,700	20,000	6,400	1,900	900
Dec	26,000	1,100	9,500	3,800	400	200
Jan 71	11,000	686	1,900	104	203	125
Feb	21,000	495	4,400	1,700	178	70
Mar	14,000	415	2,300	1,400	178	99
Apr	31,000	565	5,700	1,400	252	49
May	43,000	960	8,500	1,500	47	14
June	4,500	306	63	16	2	1
July	9,300	500	2,700	375	82	13
Mean 70-71[b]	76,000	8,700	29,000	7,700	1,500	558

[a]Count/ml river water.

[b]Means have been rounded off to the first significant digit.

Table 19. Mean CHN analysis of suspended solids present in river water from sites 1, 2 and 3

Date	Site	Carbon		Hydrogen		Nitrogen		Inert	
		%	mg/mg solid	%	mg/mg solid	%	mg/mg solid	%	mg/mg solid
1968-69	1	24.15	0.1571	2.35	0.0157	0.58	0.0035	72.92	0.4726
	2	23.22	0.1584	2.48	0.0162	0.51	0.0031	73.79	o.4539
	3	32.14	0.2178	2.57	0.0174	0.58	0.0039	64.71	0.4175
1969-70	1	23.33	0.1566	2.93	0.0209	0.58	0.0039	73.24	0.4824
	2	24.77	0.1831	2.85	0.0196	0.58	0.0041	71.88	0.4833
	3	33.38	0.2338	2.93	0.0181	0.74	0.0057	62.95	0.4205
1970-71	1	33.23	0.2000	2.69	0.0176	0.74	0.0048	63.34	0.4526
	2	33.93	0.2036	2.70	0.0187	0.79	0.0053	62.58	0.4680
	3	34.69	0.2219	2.67	0.0169	0.83	0.0070	61.81	0.4315

Table 20. Gas production by enteric bacteria recovered from river water on M-Endo MF medium at 44.5 C

Parr's IMViC[a] group	Coliform isolates[b]			Enteric isolates[c]			Total isolates		
	Organisms recovered	Gas[d]	Per cent (group total)	Organisms recovered	Gas	Per cent (group total)	Organisms recovered	Gas	Per cent (group total)
Escherichia group (++--, +---, -+--)	226	221	97.8	86	77	89.5	312	298	95.5
Enterobacter group (--++, --+-, ---+)	130	23	17.7	28	15	53.6	158	38	24.0
Intermediate group	86	44	51.2	73	66	90.4	159	110	69.2

a. Indole, methyl red, Voges-Proskauer, citrate reactions observed.

b. Organisms exhibiting lactase utilization and a metallic, green sheen.

c. Organisms exhibiting lactose utilization.

d. Production of gas in MR-VP medium at 44.5 C.

and having + + - -, - + - -, or + - - - IMViC reactions may be considered to be Escherichia species and of fecal origin, whereas - - + +, - - + -, or - - - + strains, which lack the ability to produce gas at 44.5 C, are Enterobacter and are probably of soil or vegetative origin.

The results of these experiments (Table 20) show that 70.9% of all organisms recovered produce gas, which proved to be a mixture of H_2 and CO_2, but less than 50% of the isolates were Escherichia species. Of all Enterobacter, 24% also had the capacity for gas production. Much the same correlation pattern of gas production at 44.5 C with IMViC types was observed when only the "coliform" isolates were considered. Of significant importance is the observation that 17.7% of the Enterobacter species in the coliform group produced gas from glucose.

Formate and lactose were observed to serve satisfactorily as substrates for gas production by those aquatic Enterobacter species which could produce gas at 44.5 C from glucose. The aquatic strains and E. coli could produce gas from the three substrates at both 30.0 and 44.5 C, whereas the ATCC E. aerogenes culture could not at the higher temperature. These data indicate that the evolved CO_2 and H_2 result from an active formic hydrogenlyase and that this particular enzyme system can be functional in Enterobacter at the temperature specified for the Eijkman procedure (Table 21).

Data from the Warburg experiments (Fig. 11) suggest that formic hydrogenlyase is synthesized by both the E. coli and E. aerogenes stock cultures at 30.0 C and by E. coli at 44.5 C. The induction time for formic hydrogenlyase synthesis by the stock cultures was about 30 min (Fig. 11) in each case, but when coliform organisms recovered from the aquatic environment were used in similar experiments, times of induction were variable and ranged from 30 min to approximately 2 hr. (Fig. 12). None of the aquatic organisms used in these experiments could be biochemically grouped with the Escherichia species, but all organisms used, with the exception of one (+ - + +), synthesized formic hydrogenlyase. These included an organism selected from the 23 gas-producing E. aerogenes strains. Evidence for formic hydrogenlyase induction was further demonstrated by the lack of enzymatic activity in control experiments, in which chloramphenicol was added to the substrates for induction. These data indicate that the formic hydrogenlyase system was induced and not simply an activation of the system which might have been present.

Table 22 shows specific activity of the synthesis of formic hydrogenlyase

Table 21. Gas production from glucose, lactose, and formate at 30.0 C and 44.5 C by aquatic forms of Enterobacter aerogenes conforming to the "coliform" designation[a]

Organism	No. of Strains	Source	Gas production from					
			Glucose		Lactose		Formate	
			30.0 C	44.5 C	30.0 C	44.5 C	30.0 C	44.5 C
Enterobacter aerogenes								
--++	1	ATCC 12658	+	-	+	-	+	-
--++	12	Oconee R.	+	+	+	+	+	+
--+-	3	Oconee R.	+	+	+	+	+	+
---+	8	Oconee R.	+	+	+	+	+	+
Escherichia coli	1	ATCC11775	+	+	+	+	+	+

[a]These organisms produced gas in MR-VP medium at 44.5 C.

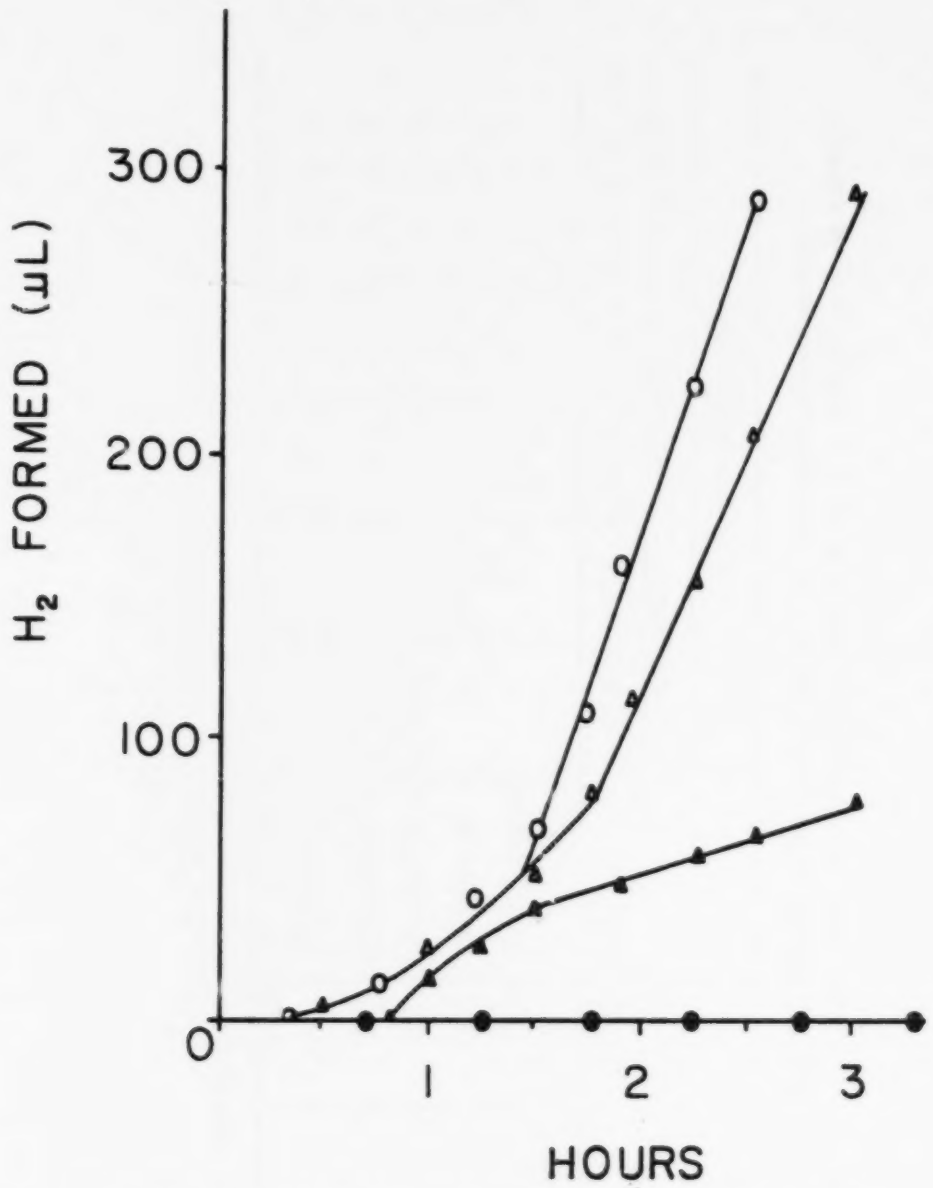


Fig. 11. Formic hydrogenlyase synthesis by Escherichia coli ATCC 11775 at 30.0 C (△), 44.5 C (▲), and Enterobacter aerogenes ATCC 12658 at 30 C (O), 44.5 C (●).

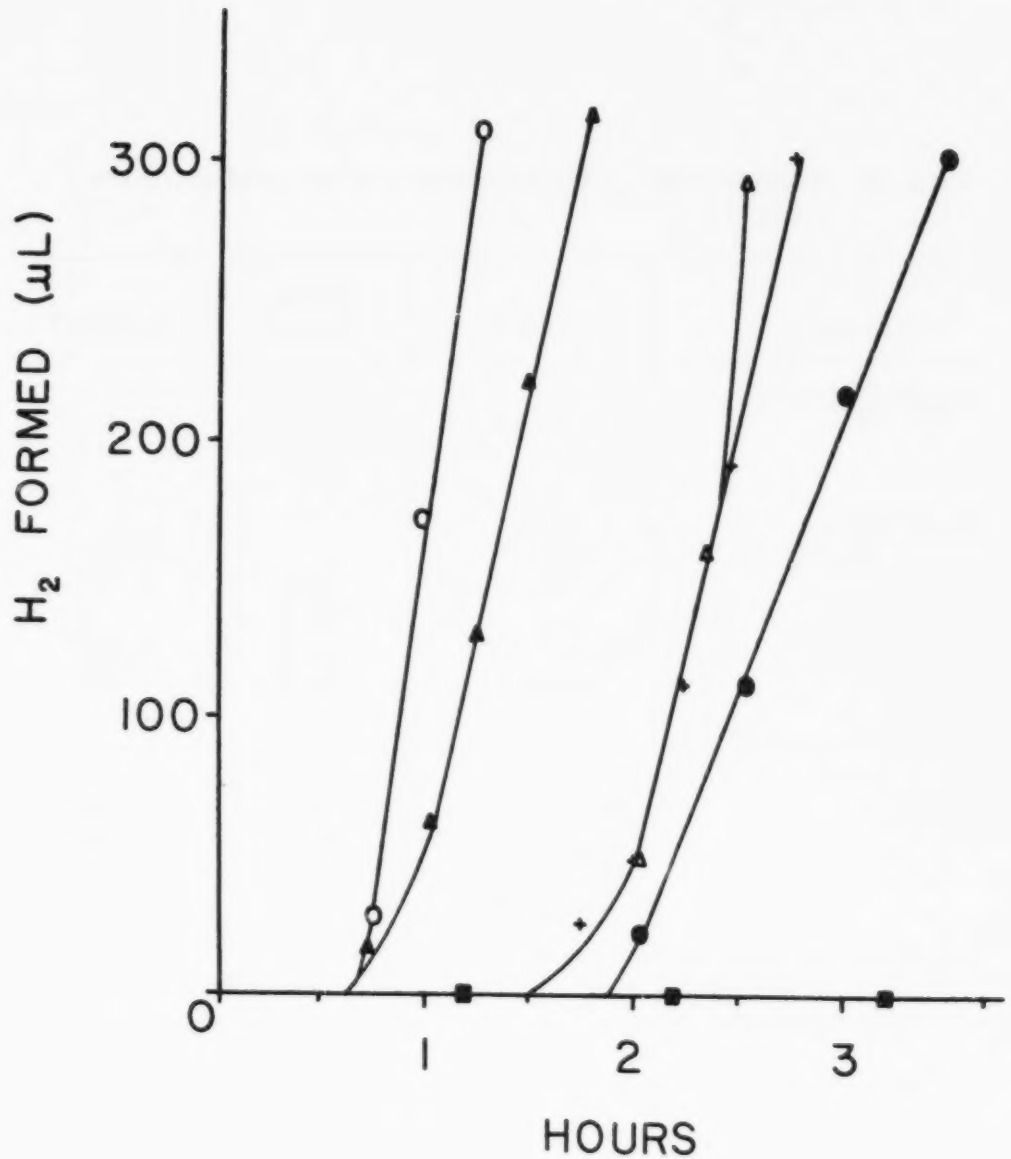


Fig. 12. Formic hydrogenlyase synthesis at 44.5 C by various enteric bacteria. Symbols used refer to the indol, methyl red, Voges-Proskauer, citrate classification of the bacteria: ▲, --+-; △, --++; ●, -+-+; O, +++-; ■, +-++; +, -+-+.

Table 22. Formic hydrogenlyase activity of selected coliform bacteria

Organism	Source	Assay Temp C	Activity[a]
Escherichia coli			
++--[b]	ATCC 11775	30.0	2,360
		44.5	360
Enterobacter aerogenes			
--++	ATCC 12658	30.0	2,680
		44.5	0
--++	Oconee R.	44.5	2,860
--+-	Oconee R.	44.5	2,500
Intermediate coliform group			
++?-	Oconee R.	44.5	3,300
-++-	Oconee R.	44.5	2,250
-+-+	Oconee R.	44.5	2,260
+-++	Oconee R.	44.5	0

[a] Expressed in microliters of H_2 per hour per milligram (dry weight) of cells.

[b] Parr's IMViC grouping.

by using data from the Warburg experiments. Known stock cultures of E. coli produced approximately 10% of the activity at the elevated temperature, whereas E. aerogenes failed to synthesize the enzyme. However, at 44.5 C, the aquatic strains of E. aerogenes were capable of synthesizing formic hydrogenlyase at a rate comparable to the stock culture at the lower temperature. In general, the intermediate forms were quite active at 44.5 C and formed formic hydrogenlyase at a rate equivalent to that of the stock cultures at 30.0 C.

Increased recovery of salmonellae from stream bottom sediments vs. surface waters: The data presented in Table 23 demonstrate that higher recovery yields of Salmonella can be achieved from bottom sediments than from surface waters at site C (see Fig. 5). Of the 195 samples of sediments and river water taken over a 1-year period (1968-1969), approximately 90% of the Salmonella species recovered were found in the bottom sediments. Explanations for this observation are difficult since a variety of both physical and biological phenomenon could be responsible for the recovery. It is entirely possible that sedimentation and sorption of the organisms to the sand and clays could concentrate bacteria on the stream bottom. This phenomenon could in itself increase the recovery yields of any desired bacterial species and, if the organism could find suitable nutrients present, growth might occur to further increase recovery yields. It is interesting to note that 8 of the 10 Salmonella were recovered when mid-day water temperatures were above 24.0 C, since we have demonstrated in the laboratory that prototrophic strains of Salmonella senftenberg and Shigella flexneri can metabolize substrates present in aqueous extracts of bottom sediments at this temperature and below (see parts 2 and 3 of this report). Although procedures used in this study were adequate for the recovery of shigellae, none could be detected on the primary isolation media from any of the study sites.

Part 2

Evaluation of the Ability of Enteric Bacteria to Use Natural Aquatic Substrates by Oxygen Uptake Experiments

Enteric bacterial metabolism of substrates in river water and in stream sediment eluates: Preliminary experiments indicated that 0.3 M phosphate buffer (pH 7.0) eluted the basal nutrients from the stream sediments in maximum concentration (Fig. 13). Table 24 presents data from experiments to determine the basic nutrient concentration of both the river water and extracts of sediments from each study site. Attempts to elute

Table 23. *Salmonella* recovered from water and bottom sediments of the North Oconee River[a]

Source	Samples taken	*Salmonella* recovered		*Salmonella* sp. isolated
		No.	Per cent	
River water	195	1	0.6	
Site C	39	1	2.6	1 *S. enteritidis* ser. *anatum*
Bottom sediments	195	9	4.6	
Site C	39	8	20.1	3 *S. enteritidis* ser. *anatum* 4 *S. enteritidis* ser. *indiana* 1 *S. enteritidis* ser. *meleagridis*
Site E	39	1	2.6	1 *S. enteritidis* ser. *indiana*

[a] Thirty-nine samples of water and of sediments were recovered from each of the five sites (195 samples each total).

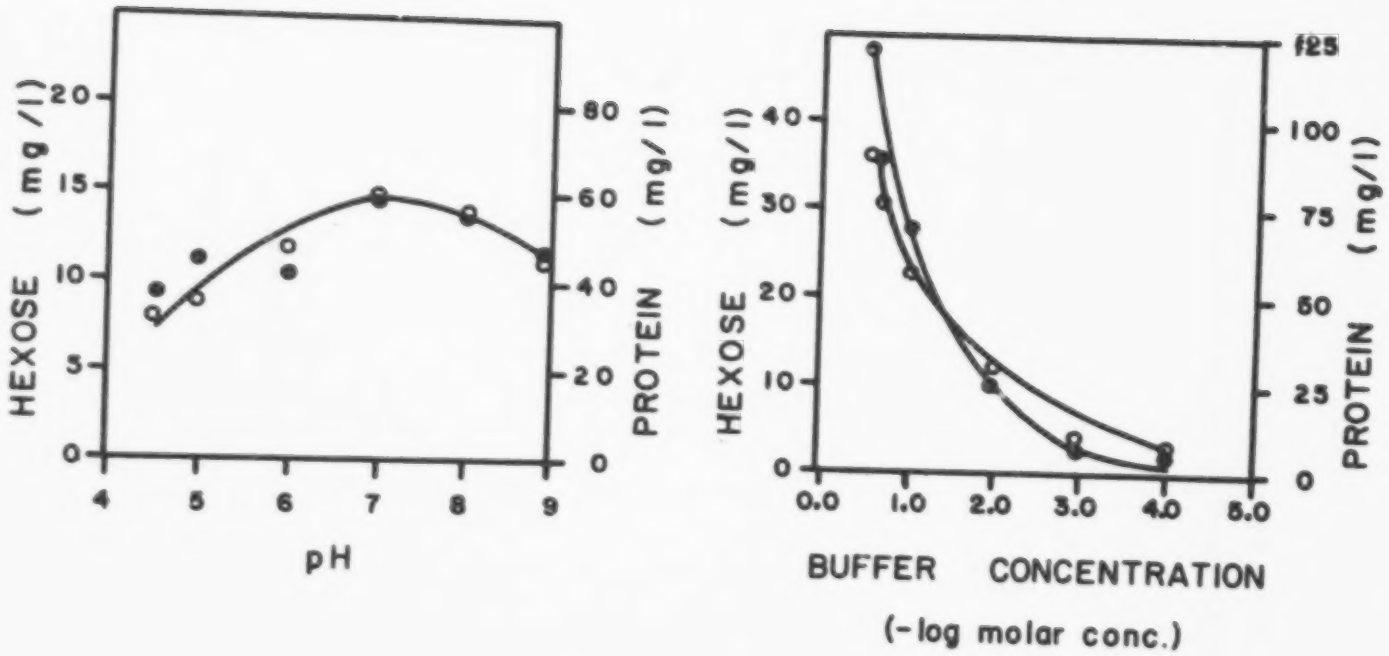


Fig. 13. Effect of pH and buffer concentration for elution of hexose (O) and protein (●) from river sediment. The pH was standardized to 7.0 in studies to determine optimal buffer concentration for maximal elution.

Table 24. Basic nutrient analysis of Oconee River water and extracts of river bottom sediment from the three study sites

Nutrient[a] assayed	Site	River water	Bottom sediment	
			Washed[b]	Buffer eluted[c]
Ammonium nitrogen	1	1.6	0.6	9.0
	2	2.3	0.2	8.5
	3	4.7	1.0	25.0
Folin protein	1	13.6	1.5	45.0
	2	13.8	0.2	63.0
	3	18.3	0.2	120.0
Hexose	1	1.6	1.0	12.0
	2	2.8	0.6	22.5
	3	1.0	2.4	36.0
Orthophosphate	1	1.5	0.0	-[d]
	2	2.0	0.0	-
	3	4.1	0.4	-
pH	1	6.9	7.0	7.0
	2	7.0	7.0	7.0
	3	7.0	7.0	7.0

[a] Concentrations expressed in mg/l of sample.

[b] Concentration of nutrients present in the 3rd successive washing of deionized water.

[c] Sediment eluted with 0.3 M phosphate buffer (pH 7.0).

[d] Phosphate concentrations were 0.3 M.

measurable quantities of the basal nutrients from the sediments with river water above those already present in the river water were without success.

Control experiments demonstrating basal respiration levels by the nonpathogenic enteric bacteria (E. coli, E. aerogenes, and P. rettgeri) with diluted minimal inorganic salts - glucose media are presented in Fig. 14. No uptake of oxygen was observed when the test organisms were run in deionized water before each respiration rate determination, and no apparent phosphate effect upon respiration was observed at the final experimental concentration level of 0.2 M.

Use of the substrates present in the river water and in washed and buffer eluted bottom sediments is shown in Tables 25 through 27 and in Fig. 15. Tables 25, 26 and 27 express the respiration rates of the nonpathogenic enterics at 30, 20 and 5 C with the test substrates from sites 1, 2 and 3 respectively. In Fig. 15 are compared the respiration rates of the nonpathogenic enterics with pathogenic species of S. flexneri, S. senftenberg, and A. arizonae in buffer eluted sediments and river water from below the sewage plant (site 3).

Utilization of substrates in detritus samples: Table 28 demonstrates the results obtained when suspended and concentrated stream detritus samples were used as substrates for metabolism by coliform bacteria. Slime (polysaccharide capsular material) produced by E. aerogenes was used as a control substrate.

The device of Pomeroy and Johannas (1968) was an effective means for concentrating the detritus used in this portion of the study. A 5.7 fold increase in concentration over the suspended (lyophilized) detritus being observed, while the overall concentration factor for hexose in the concentration detritus as compared to river water was 61 fold.

Part 3

Evaluation of the Ability of Enteric Bacteria to Use Natural Aquatic Substrates by Continuous Culture Experiments

Preliminary experiments: The basal nutrient concentration of the river water from site 3 and that of the control substrate dilute minimal medium is presented in Table 29. Sufficient river water was collected and processed before the studies were initiated so that the basal nutrient concentration for each experiment was identical. Figures 16 and 17 show results of short term continuous culture experiments (24 hrs.)

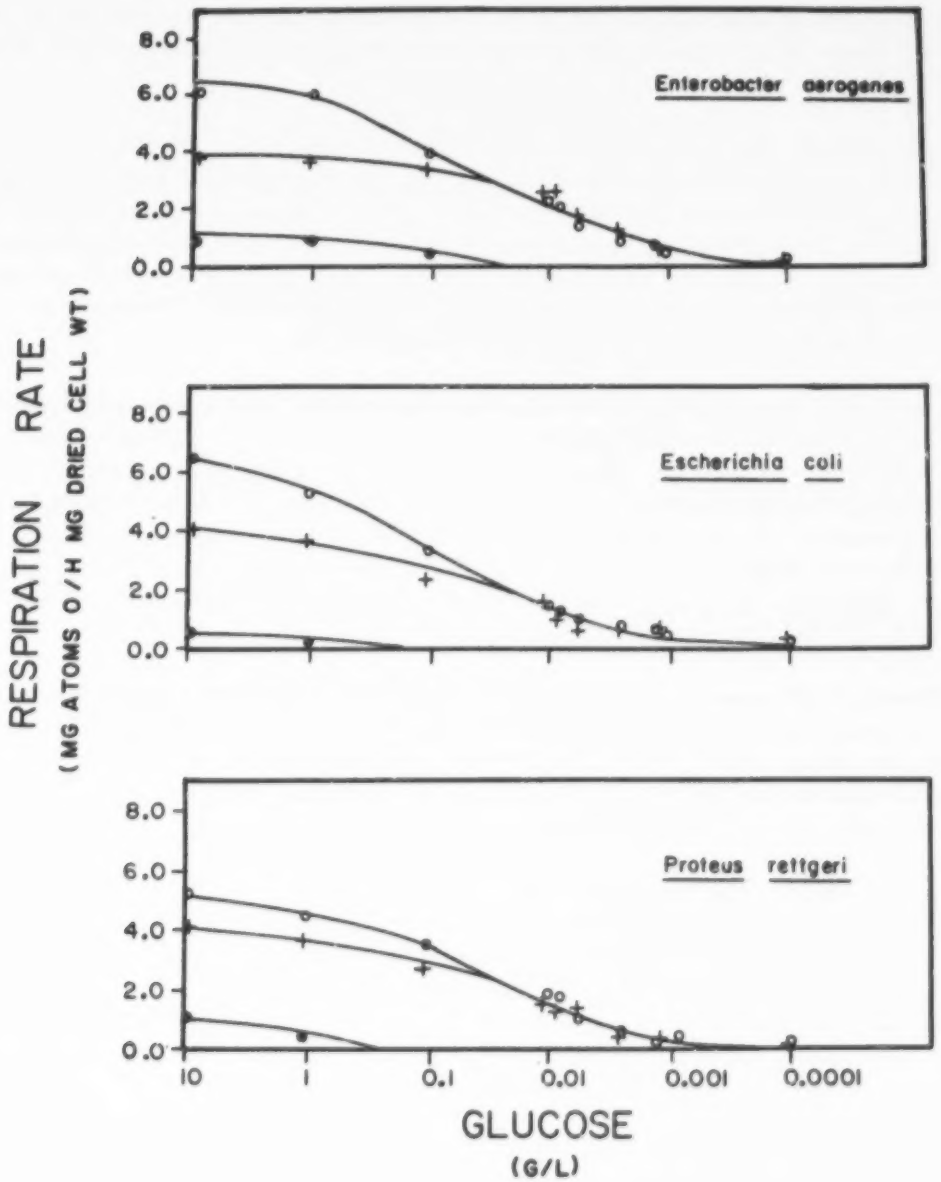


Fig. 14. Respiration of selected enteric bacteria in minimal inorganic salts medium containing various concentrations of glucose at 30 C, (O); 20 C, (+); and 5 C, (●). Respiration levels have been corrected for endogenous activity.

Table 25. Respiration of various enteric bacteria in Oconee River water and in extracts of river bottom sediments from site 1

Organism	Temp. C	River water[a]	Bottom sediment	
			Washed[b]	Buffer eluted[c]
Escherichia coli	30	0.19[d]	0.34	1.98
	20	0.00	0.00	0.44
	5	0.00	0.00	0.44
Enterobacter aerogenes	30	0.22	0.46	1.60
	20	0.00	0.09	1.13
	5	0.00	0.16	0.00
Proteus rettgeri	30	0.26	0.12	1.64
	20	0.00	0.00	1.38
	5	0.00	0.00	0.00

[a]Respiration rates expressed as mg atoms oxygen (O)/h mg dry cell weight.

[b]After third successive wash.

[c]0.3 M phosphate buffer (pH 7.0).

[d]All respiration rates have been corrected for endogenous activity.

Table 26. Respiration of various enteric bacteria in Oconee River water and in extracts of river bottom sediments from site 2

Organism	Temp. C	River water[a]	Bottom sediment	
			Washed[b]	Buffer eluted[c]
Escherichia coli	30	0.43[d]	0.23	1.81
	20	0.21	0.52	1.68
	5	0.00	0.00	0.18
Enterobacter aerogenes	30	0.53	0.37	1.26
	20	0.00	0.00	1.27
	5	0.00	0.24	0.10
Proteus rettgeri	30	0.25	0.00	1.68
	20	0.00	0.21	0.80
	5	0.00	0.00	0.00

[a]Respiration rates expressed as mg atoms oxygen (O)/h mg dry cell weight.

[b]After third successive wash.

[c]0.3 M phosphate buffer (pH 7.0).

[d]All respiration rates have been corrected for endogenous activity.

Table 27. Respiration of various enteric bacteria in Oconee River water and in extracts of river bottom sediments from site 3

Organism	Temp. C	River water[a]	Bottom sediment	
			Washed[b]	Buffer eluted[c]
Escherichia coli	30	0.58[d]	0.22	2.58
	20	0.33	0.10	1.73
	5	0.00	0.00	0.34
Enterobacter aerogenes	30	0.45	1.34	4.25
	20	0.00	0.90	1.13
	5	0.00	0.00	0.10
Proteus rettgeri	30	0.36	0.36	3.23
	20	0.17	0.12	1.07
	5	0.00	0.00	0.00

[a] Respiration rates expressed as mg atoms oxygen (O)/h dry cell weight.

[b] After third successive wash

[c] 0.3 M phosphate buffer (pH 7.0).

[d] All respiration rates have been corrected for endogenous activity.

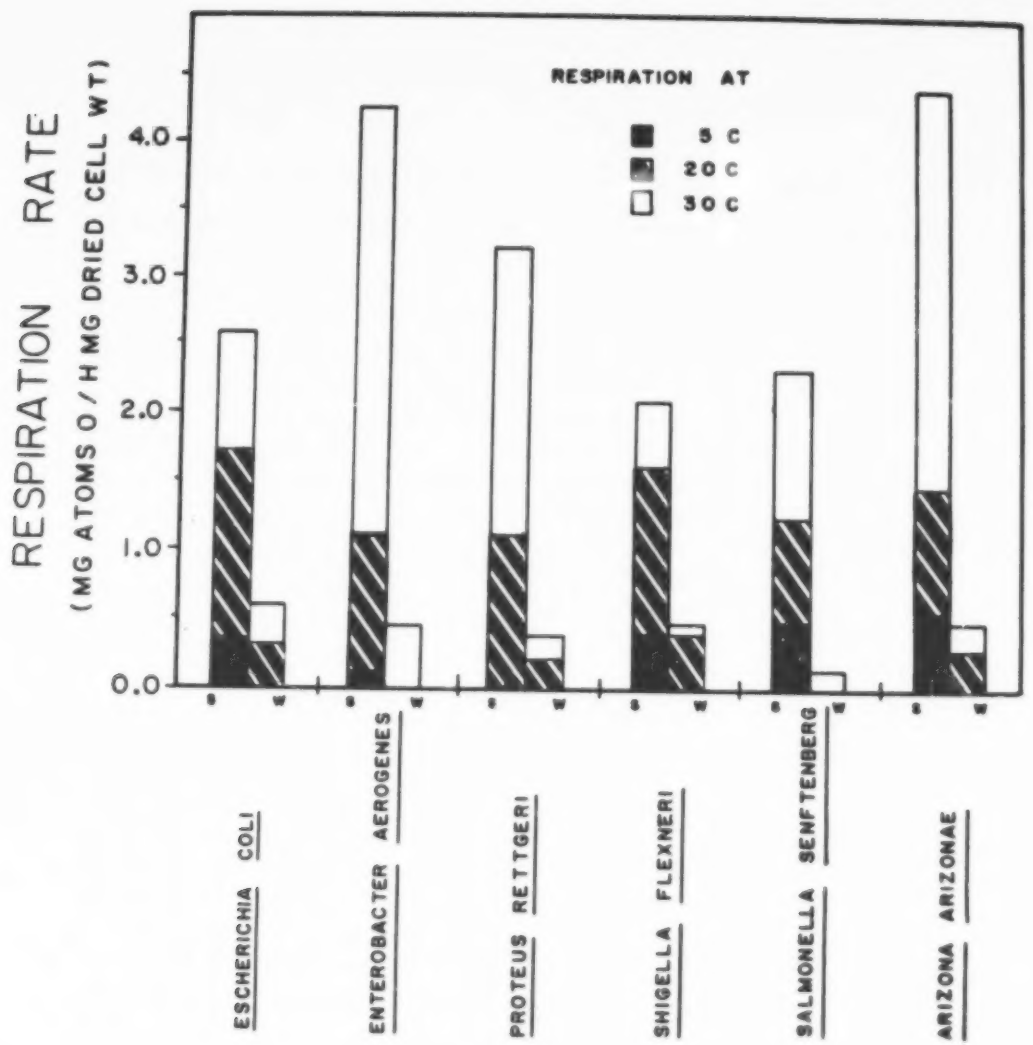


Fig. 15. Respiration rates of selected pathogenic and nonpathogenic enteric bacteria in 0.3 M phosphate buffer extracted sediments (S) and river water (W).

Table 28. Respiration rates of Enterobacter aerogenes (ATCC 12658) and Escherichia coli (ATCC 11775) using slime and stream detritus as substrates

Substrate	Hexose concentration (μg/ml)	Respiration Rate (mgO/hr. /mgdcw[a])		Respiration Rate/μg hexose	
		E. aerogenes	E. coli	E. aerogenes	E. coli
Slime[b]	31.8	0.63	0.10	0.020	0.003
Concentrated detritus	116.0	3.77	2.80	0.033	0.024
Suspended detritus (lyophilized)	28.4	0.95	0.10	0.033	0.006
Suspended detritus (oven dried[c])	38.2	1.27	0.40	0.033	0.010

[a] mgO/hr. /mgdcw = milligrams molecular oxygen per hour per milligrams of dried (105 C for 24 hrs) cell weight.

[b] Polysaccharide capsular material of E. aerogenes.

[c] Dried at 105 C for 24 hours.

TABLE 29. Basal nutrient concentration of autoclaved site 3 river water and dilute minimal salts-glucose medium[a]

Assay	Source	Concentration Mg/l
Ammonia nitrogen	Site 1	1.1
	Site 2	1.5
	Site 3	3.0
	Minimal Medium	0.5
Protein	Site 1	2.9
	Site 2	3.2
	Site 3	5.1
	Minimal Medium	0.0
Hexose	Site 1	2.1
	Site 2	2.4
	Site 3	2.8
	Minimal Medium	10.0
Phosphate	Site 1	1.3
	Site 2	1.6
	Site 3	3.2
	Minimal Medium	5.0
pH	Site 1	7.0
	Site 2	6.9
	Site 3	6.9
	Minimal Medium	7.0

[a]Minimal medium diluted 1:1,000.

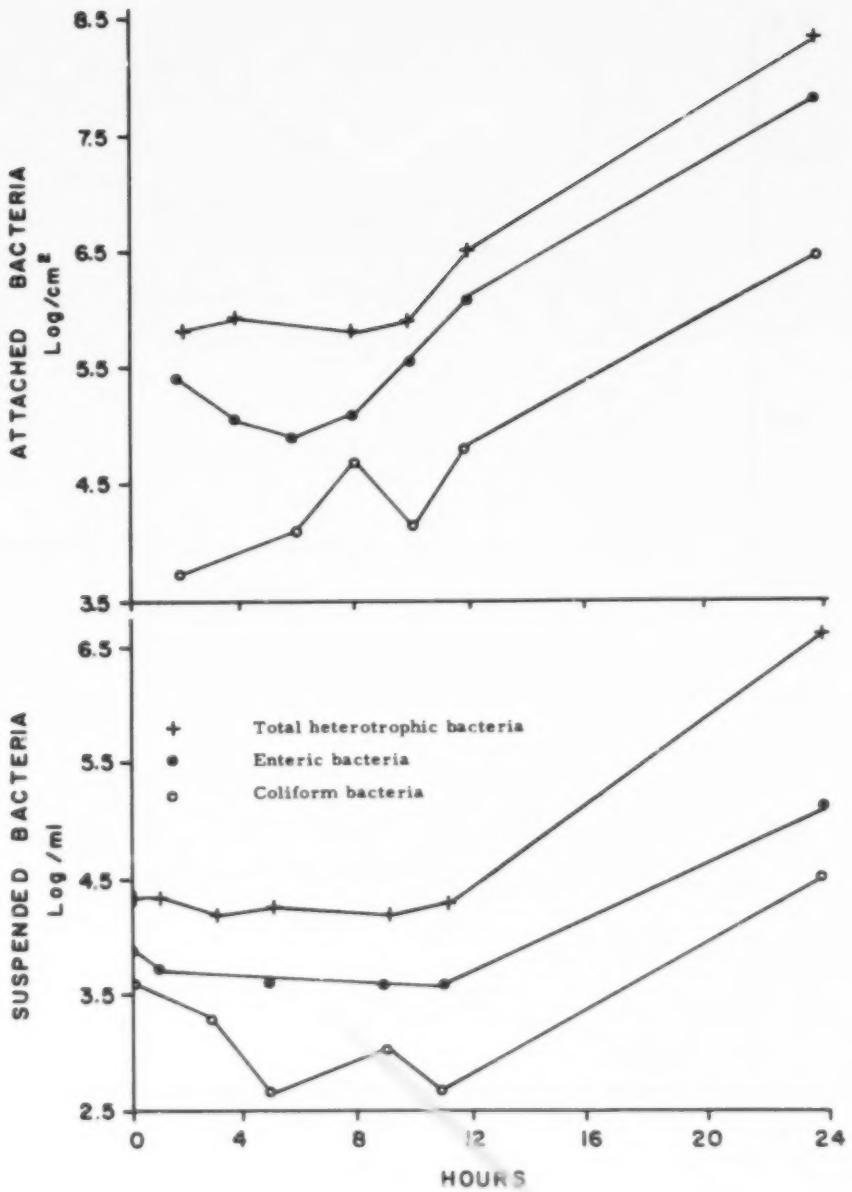


Fig. 16. Continuous culture of a natural heterotrophic bacterial population in Oconee River water from site 3 at 14 C. Dilution rate = 0.058 hr.$^{-1}$.

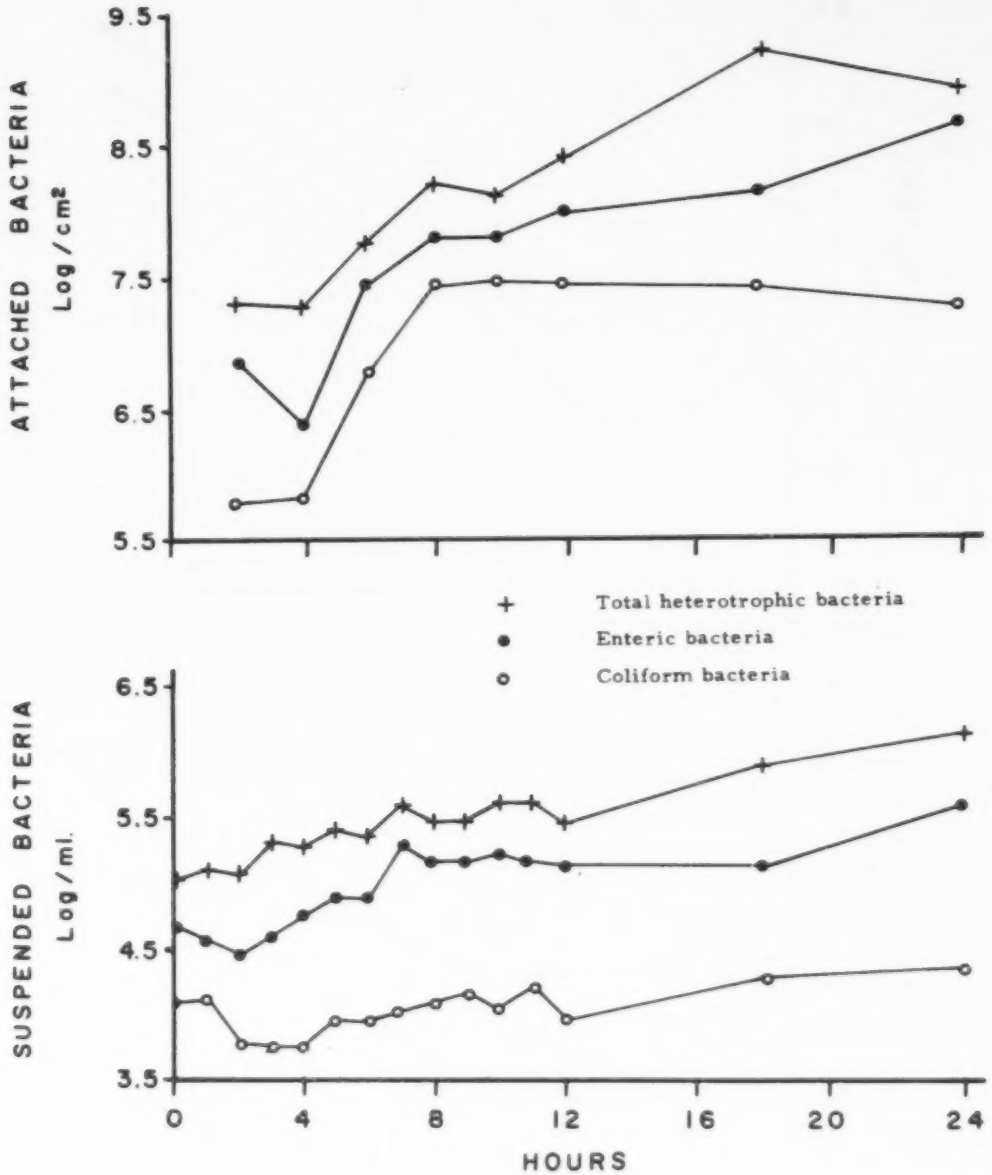


Fig. 17. Continuous culture of a natural heterotrophic bacterial population in Oconee River water from site 3 at 26 C. Dilution rate = 0.058 hr.$^{-1}$.

conducted at 14 and 26 C respectively in site 3 river water and at dilution rate of 0.58 hr.$^{-1}$. Over the 24 hour period, oxygen concentration was reduced 12% in the 14 C experiment and 50% in the device run at 26 C. Basal nutrient concentrations in both series of experiments did not appear to fluctuate greatly and showed only a slight decrease in hexose and ammonia nitrogen content, no change in phosphate and an elevated protein concentration as compared to the nutrient concentration in the resevoir. Figure 19 contains results from 420 hr. continuous culture experiment using dilute minimal salts and glucose as nutrient source, while Fig. 18 shows the results of a similar experiment with river water as a nutrient source. Both of these experiments were run at dilution rates of 0.012 hr.$^{-1}$ and incubation temperatures of 30 C. Table 30 shows the growth rates for these experiments.

Prior to the initiation of the continuous culture experiments using the six enteric test strains, a standard dilution rate of 0.012 hr.$^{-1}$ was found to yield an adequate growth rate for E. coli in site 3 river water culture experiments at 30 C (Fig. 20). This flow rate was used in all subsequent continuous culture experiments at each of the three standard incubation temperatures (30, 20 and 5 C).

Continuous culture experiments using river water and the collected detritus from the three sites as substrates: River water from sites 1 and 2, in each case, failed to provide any of the six test organisms with sufficient nutrients to allow for growth at the three selected temperatures. Table 31, however, contains data on growth rates of the six bacterial test strains in water from site 3.

In the continuous culture experiments using both concentrated and suspended detritus, no significant growth for E. coli was observed at 20 or at 5 C, but low growth rates were observed at 30 C for this organism (Table 32). Attempts to determine why higher growth rates were not observed in these experiments demonstrated that basal nutrients had adsorbed to the inorganic particulate material which has formed a part of the concentrated detritus sample (Table 33).

Bdellovibrio bacteriovorus - Escherichia coli interactions

Activity in river water: A typical growth curve at 30 C for B. bacteriovorus in NB Medium is presented in Fig. 21, while Fig. 22 shows the same experiment using river water from site 3 as a nutrient source. Figure 23 is the result of an experiment designed to demonstrate that if additional nutrients are added to E. coli-B. bacteriovorus system, numbers of both organisms would be stimulated. Results of

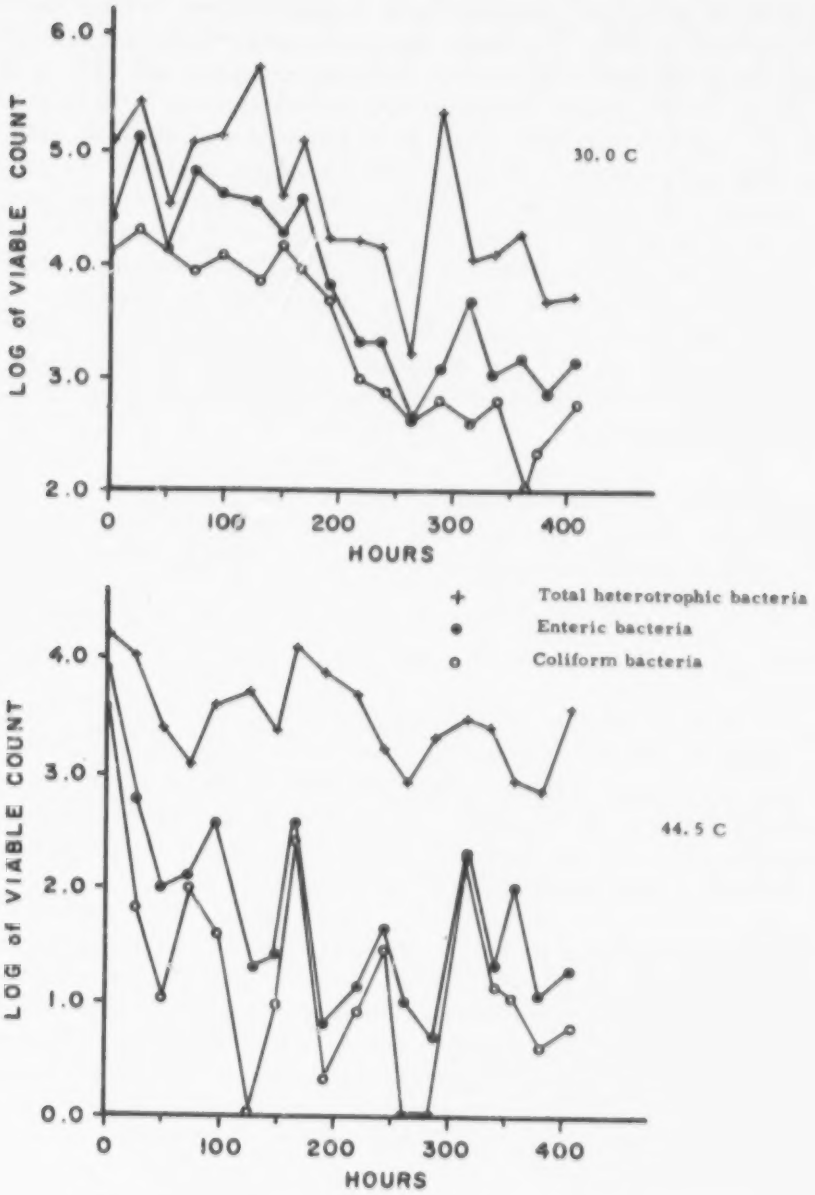


Fig. 18. Continuous culture of a natural heterotrophic bacterial population in Oconee River water from site 3 at 30 C. Dilution rate = 0.012 $hr.^{-1}$.

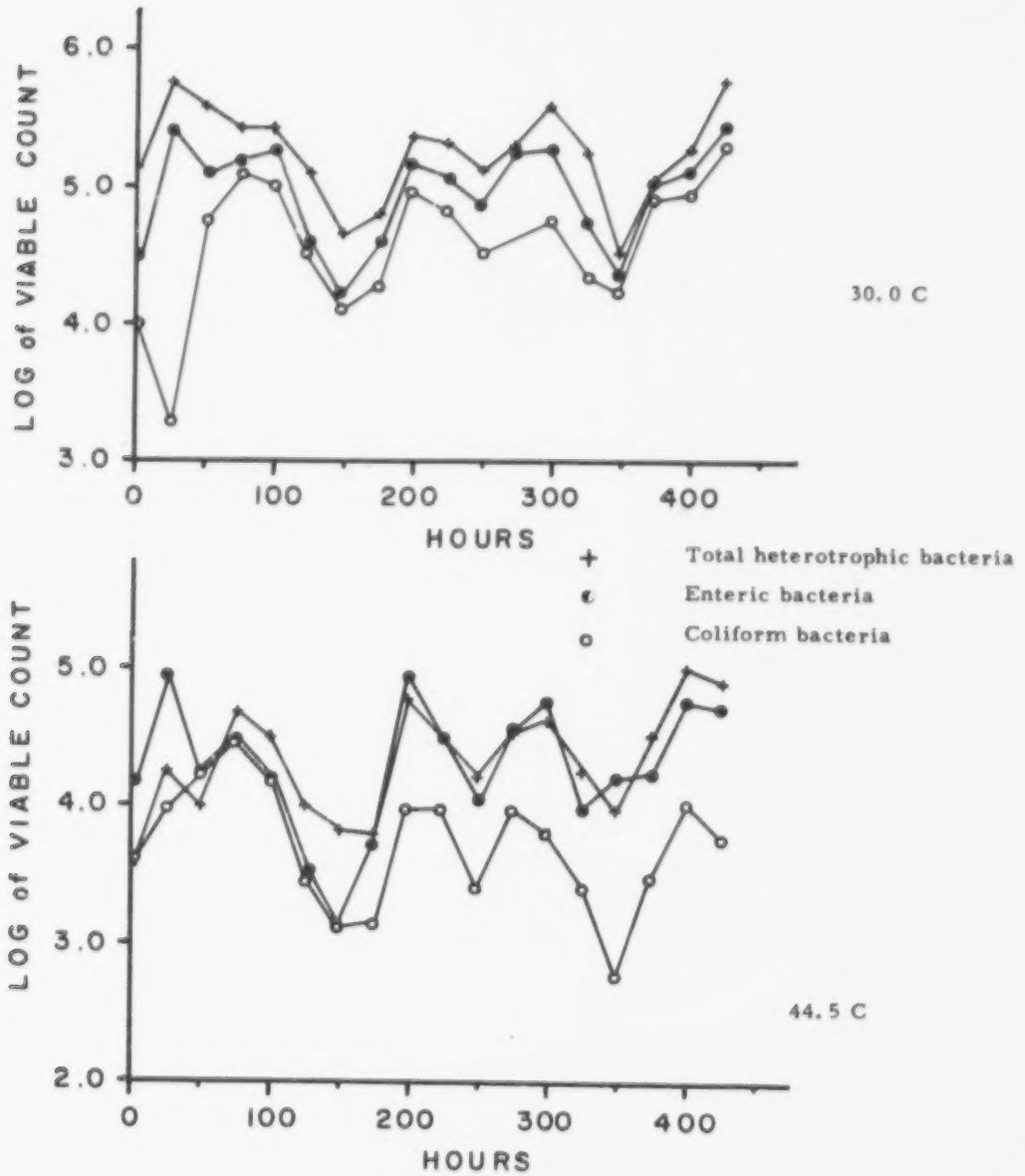


Fig. 19. Continuous culture of a natural heterotrophic bacterial population at 30 C in minimal salts medium diluted 1:1000. Dilution rate = 0.012 hr.$^{-1}$.

Table 30. Average growth rates of native bacterial populations in site 3 Oconee River water and dilute minimal medium

Nutrient Source	Bacterial Group	Growth Rate hr.$^{-1}$	
		30 C Population	44.5 C Population
Minimal Medium 1:1,000 dilution (Davis, 1950)	Heterotrophs	0.017	0.019
	Enterics	0.018	0.015
	Coliforms	0.019	0.012
River Water (Site 3)	Heterotrophs	0.007	0.013
	Enterics	0.007	d[a]
	Coliforms	0.006	d

[a] Death of the culture

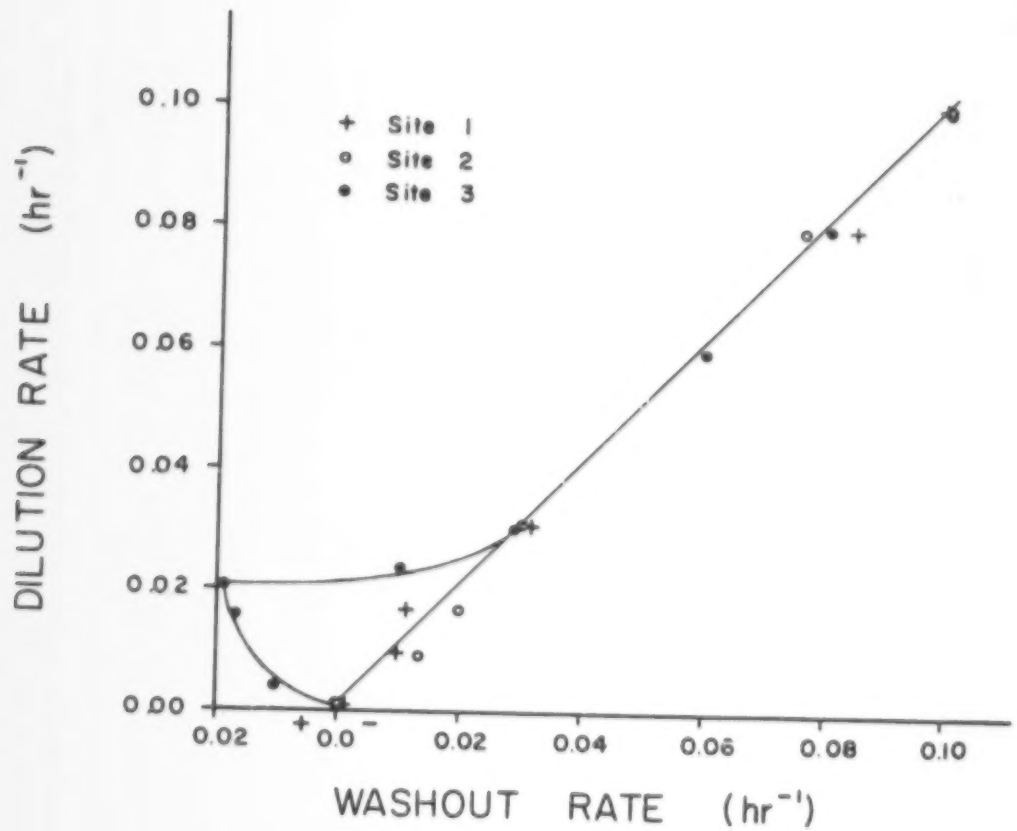


Fig. 20. Washout rate vs. dilution rate plot for Escherichia coli in Oconee River water from sites 1, 2, and 3 at 30 C.

Table 31. Growth rates[a] of selected enteric bacteria in Oconee River water from site 3

Organism	Temperature C	Wash out Rate ($hr.^{-1}$)	Growth Rate ($hr.^{-1}$)	Generation Time (hrs.)
Escherichia coli	30	0.017	0.029	34.5
	20	-0.009	0.003	333.3
	5	-0.011	0.001	1,000.0
Enterobacter aerogenes	30	0.018	0.030	33.3
	20	-0.006	0.006	166.6
	5	-0.018	-	-
Proteus rettgeri	30	-0.002	0.012	83.3
	20	-0.001	0.010	100.0
	5	-0.017	-	-
Arizonae arizona	30	-0.001	0.011	90.0
	20	-0.011	0.001	1,000.0
	5	-0.011	0.001	1,000.0
Salmonella senftenberg	30	0.001	0.013	76.9
	20	-0.020	-	-
	5	-0.032	-	-
Shigella flexneri	30	0.001	0.013	76.9
	20	-0.007	0.005	200.0
	5	-0.010	0.002	500.0

[a] Dilution rate was 0.012 $hr.^{-1}$ in each case.

Table 32. Growth rate of Escherichia coli at 30 C in concentrated stream detritus from the Oconee River

Growth Parameter	Site 1	Site 2	Site 3
Retention time (hrs)	83.3	83.3	83.3
Dilution Rate (hr^{-1})	0.012	0.012	0.012
Wash out Rate (hr^{-1})	-0.011	-0.016	-0.003
Growth Rate (hr^{-1})	0.001	-0.004	0.009
Generation time (hrs)	1,000	-	111.1

Table 33. Respiration rate of Escherichia coli at 30 C in detritus samples and eluates of sediments present in detritus samples from site 3

Substrate	Respiration Rate Mg atoms O/hr. /mg dry cell wt.
Detritus Sample	1.25
Eluates	
Wash [a] 1	0.70
Wash 2	0.85
Wash 3	0.85
0.3 M PO_4 buffer	2.50

[a] Deionized water wash.

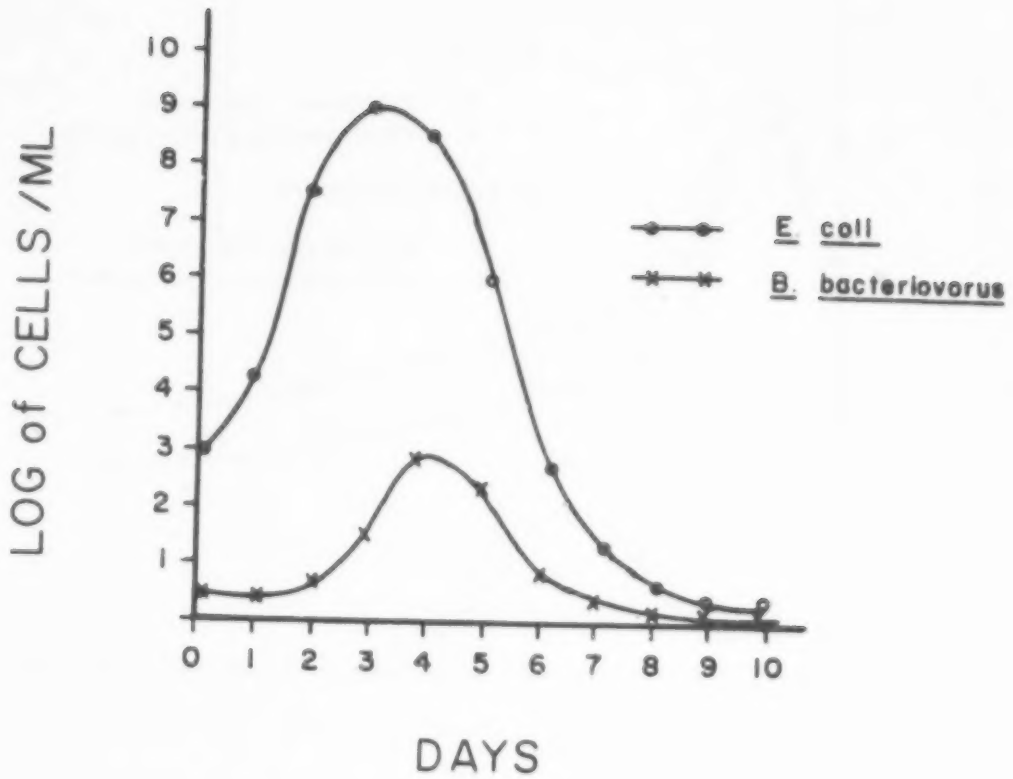


Fig. 21. Growth curve of Escherichia coli and Bdellovibrio bacteriovorus in N. B. medium at 30 C.

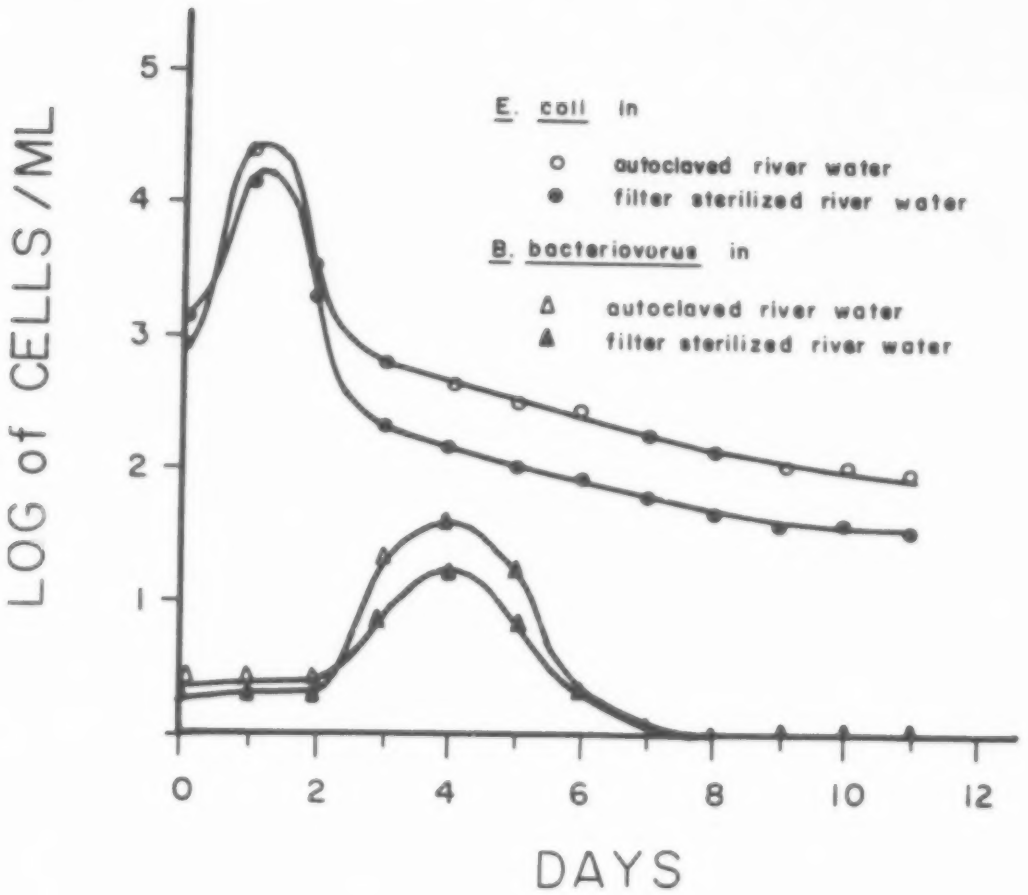


Fig. 22. Growth curve of Escherichia coli and Bdellovibrio bacteriovorus in filtered and autoclaved Site 3 Oconee River water at 30 C.

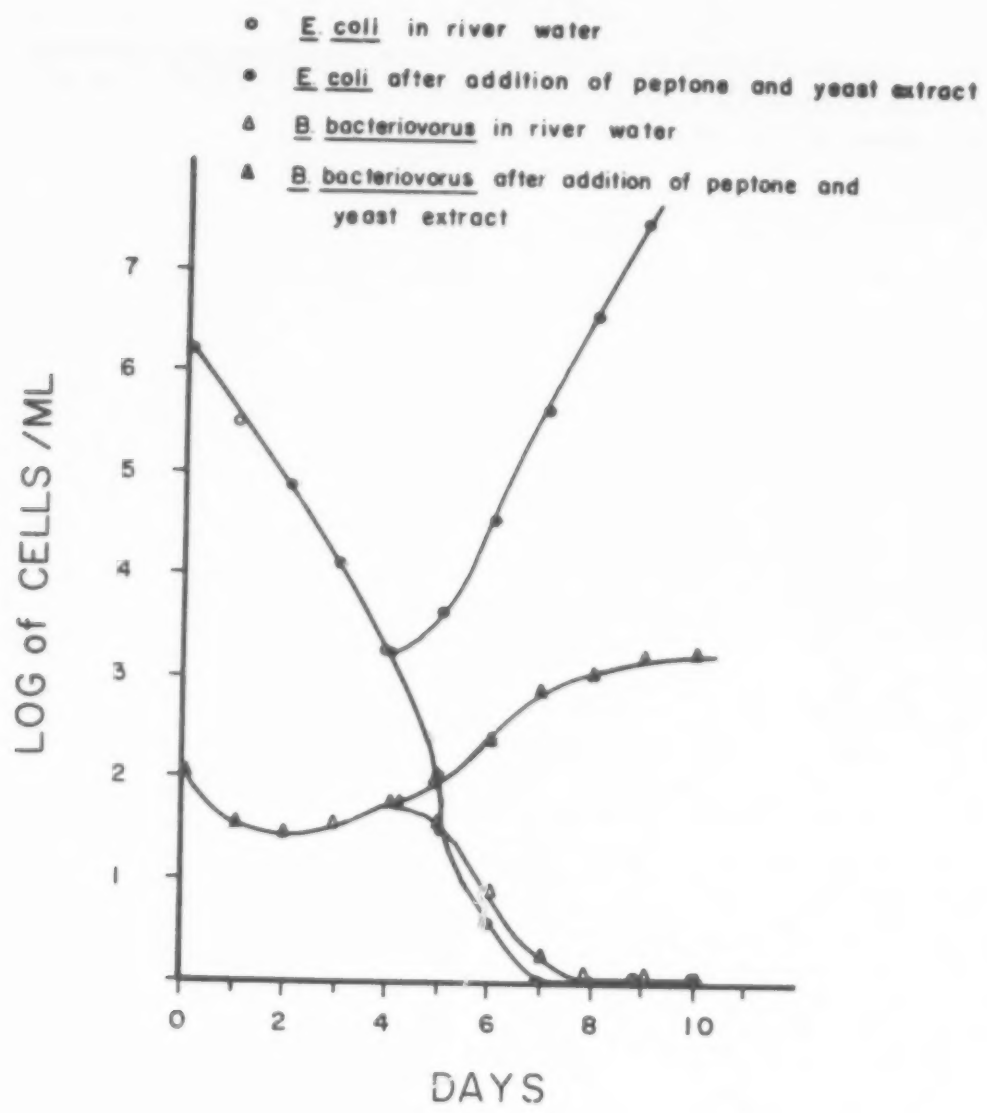


Fig. 23. Growth curve of Escherichia coli and Bdellovibrio bacteriovorus in site 3 river water at 30 C. Peptone and yeast extracts were added to the culture after 4 days.

the continuous culture of the E. coli and B. bacteriovorus parasitic system in river water from site 3 (dilution rate 0.012 hr.$^{-1}$ 30 C) are shown in Fig. 24.

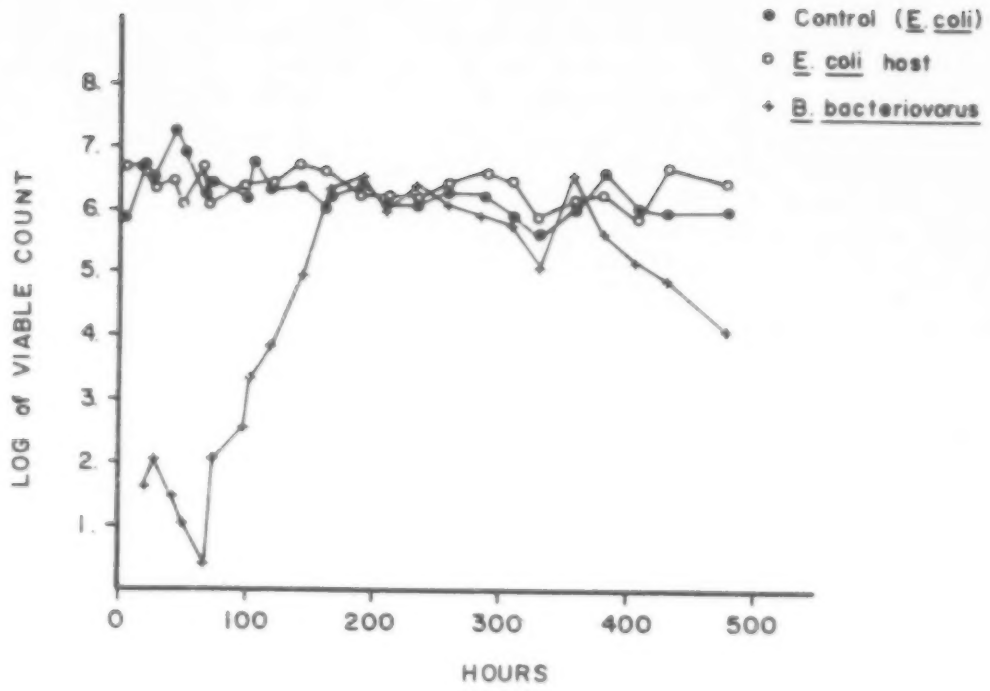


Fig. 24. Continuous culture of Escherichia coli and Bdellovibrio bacteriovorus in Oconee River water from site 3 at 30 C.

SECTION VI

DISCUSSION

From August, 1968 through July, 1971, three integrated studies were undertaken to study enteric bacterial metabolism of various substrates found in a fresh water stream environment. These studies are as follows:

1. Water quality analysis of the North Oconee River at three selected sites at Athens, Georgia.
2. Evaluation of the ability of enteric bacteria to use natural aquatic substrates by oxygen uptake experiments.
3. Evaluation of the ability of enteric bacteria to use natural aquatic substrates by continuous culture.

Part 1

Water Quality Analysis of the North Oconee River at Three Selected Sites at Athens, Georgia

Chemical and bacteriological assays: River water from the three study sites was sampled at least twice monthly and analyzed for basal nutrient concentration and for numbers and types of organisms present. Little deterioration of the Oconee River in terms of water quality was observed between sites one and two, but the most dramatic change in water quality did occur in the site three area downstream from the sewage plant effluent. The water at this location, however, does not appear to be grossly polluted at this time and perhaps the only potential hazard of an immediate nature might be from the pathogenic organisms which are present in the stream. (Table 23).

There appears to be little or no correlation between basal nutrient concentration and bacterial numbers, but this is not surprising since both determinations are "standing crop" estimations and yield little information concerning bacterial activity or turnover of the nutrients within the system.

Procedure evaluations

Formic hydrogenlyase induction as a basis for the Eijkman fecal coliform concept: Gray and Gest (1965) have postulated that the hydrogenlyase system present in Enterobacteriaceae contains two distinct enzymes, a hydrogenase and a formate dehydrogenase.

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This system is inducible and anaerobic, with carbon dioxide and molecular hydrogen as reaction end products. The E. coli and E. aerogenes cultures used in this investigation had the capacity to induce formic hydrogenlyase synthesis at 30.0 C, but E. aerogenes was unable to synthesize the enzyme system at 44.5 C. These results are consistent with those of Quist and Stokes (1969). The recovery of biochemically sound strains of E. aerogenes from the aquatic environment, which have an inducible formic hydrogenlyase system at 44.5 C, is not surprising (Table 21, 22), since biochemical, morphological and serological variations in enteric bacteria as laboratory phenomena are well known (Edwards and Ewing , 1962; Hayes, 1968). Reports of such phenomena occurring under natural conditions are rare, although Velaudapillai (1961) and Hendricks and Morrison (1967a) have suggested that exchange of genetic material between bacteria can occur in various natural environments to give rise to biochemically aberrant organisms.

Explanations for the presence of a high temperature-insensitive hydrogenlyase system in some of the aquatic strains of E. aerogenes are difficult. It is entirely possible that the wild type, as represented by this ATCC culture, cannot synthesize the enzyme system or synthesizes a portion that is inactivated at elevated temperature and that the selective pressures of the aquatic environment allow a particular mutant population to survive. This hypothesis is consistent with observations of Peterson and Gunderson (1960) and Morita and Burton (1963). It is also reasonable to suggest that a formic hydrogenlyase system active at an elevated temperature may be irreversibly lost upon prolonged storage as are virulent factors of certain pathogenic organisms.

Considerable evidence has recently been discovered which indicates that the positive correlation between gas production at an elevated temperature of incubation and the presence of fecal coliforms (E. coli) may be restricted to environments that are grossly contaminated by feces of man and certain warm-blooded animals (Geldreich et al. 1962; Mishra et al., 1968). In this particular environment, it is obvious that the gas-producing coliforms are indicative of fecal pollution, since E. coli assumes dominant proportions among those organisms capable of synthesizing an active formic hydrogenlyase. However, in environments where fecal contamination and numbers of E. coli are minimal, other gas-producing enteric bacteria, which are not fecal coliforms, can reach significant proportions to alter the statistical fecal coliform relationship. The results of this study substantiates the latter hypothesis, in which significant numbers of Enterobacter species (24%) from relatively clean river water had the capacity to produce H_2 and CO_2 at 44.5 C (Table 20).

Although Enterobacter species may be recovered from the feces of man and animals (Lofton et al., 1962), these organisms are not considered to be indicators of fecal pollution, but rather they are associated with soil and vegetation. The present data becomes significant when one considers the increasing reports (Boyd and Boyd, 1962; Hendricks and Morrison, 1967b), of growth and multiplication of the E. coli-E. aerogenes group of coliforms in natural waters. It is entirely possible that water of questionable quality may be needlessly rejected if differentiation between fecal and nonfecal coliforms is made solely on the basis of an elevated-temperature fecal coliform test.

Increased recovery of salmonellae from stream bottom sediments vs. surface waters: Although Salmonella can be recovered below sewage treatment facilities by a variety of techniques, it is especially interesting that more of these enteric pathogens can be recovered from bottom sediments than surface water by a relatively unsophisticated procedure. These data suggest that there is at least a migration of the organisms from the surface water to the bottom sediments where perhaps a more favorable condition exists for survival and later multiplication.

It is also of interest that nine Salmonella were recovered from the site C location. The lack of adequate mixing and dispersal of the organisms present in the sewage effluent with the river water was probably responsible for the lack of Salmonella recovery at the upper two sites. No explanation is readily available for lower recovery rates at sites below the C location, unless substantial precipitation and sorption of the organisms to the bottom sediments occurred in the site C area to preclude recovery downstream.

Part 2

Evaluation of the Ability of Enteric Bacteria to Use Natural Substrates by Oxygen Uptake Experiments

Enteric bacterial metabolism of substrates in river water and in stream sediment water: Previous studies (Butterfield, 1929; McGrew and Mallette, 1962; Hendricks and Morrison, 1967b) have indicated that basal nutrients in concentrations approximately equal to Davis' (1950) minimal salts-glucose medium diluted 1:1000 were sufficient for the maintenance and limited growth of prototrophic enteric bacteria. Data obtained in this study (Table 24) demonstrated that the ammonia nitrogen, carbon, and orthophosphate present in the three test substrates were comparable in concentration to those media. With the exception of hexose present in the river water, concentrations of the basal nutrients

contained in both the river water and buffer-extracted bottom sediments were maximal at the site located below the sewage outfall (site 3).

Table 24 also demonstrates that bottom sediments from the Oconee River can be washed relatively free from loosely associated material and that a very high concentration of ammonia nitrogen, protein, and hexose can be sorbed onto the river bottom sediments. This observation becomes even more significant because three successive washings with deionized water and trial elution with river water could not remove the tightly bound material. Elution, though, was accomplished with buffer of ionic strengths which might be common to only the most severely polluted aquatic environments. These data suggest that the basal nutrients were very tightly sorbed on the sands and clays forming the stream bottom sediments, and that they may not be readily available for metabolism by aquatic microorganisms. We have tested this concept with BOD studies in our laboratory and found that washed sediments had no stimulating effect on the oxygen demand. This is in agreement with Weber and Coble (1968), who have found that cationic pesticides which were subject to microbial degradation could be adsorbed on various clays and were then no longer subject to decomposition or even readily available for plant uptake.

Respiration of organisms in the aquatic environment has been used as a means of estimating in situ activity (Olson and Rueger, 1968; Rueger et al., 1968; Schroeder, 1968.) Control experiments (Fig. 13) suggest that a respiration rate of about 0.5-3.5 mg atoms 0/h/mg dried cell weight could be achieved if the carbon (hexose and protein) analyses of the river water and extract bottom sediments represent readily oxidizable substrates. Similar rates would be expected with the prepared natural substrates at both the 30 and 20 C incubation temperatures, but respiration at 5 C should be minimal or nonexistent. Results of the respiration rate studies using river water and extracted sediments confirmed this hypothesis (Table 25, 26, and 27) and reflected the basal nutrient concentrations. Respiration rates above endogenous levels for all organisms tested in river water were lowest at site 1, but as nutrient concentration increased in the water from sites 2 and 3, respiration rates at both 20 and 30 C approached the predicted values (Tables 26 and 27). Sediment eluted with phosphate buffer in all cases yielded respiration rates far exceeding those observed with river water. With the exception of Enterobacter aerogenes at site 1, both Escherichia coli and Enterobacter aerogenes could use the substrates present in the eluates from all sites at 5 C. Buffer eluted sediments from below the sewage plant (site 3) demonstrated the highest respiration rates

achieved at 30 C with rates at 20 and 5 C approximating those at the other two sites. When these rates were compared with those for the pathogenic species (Shigella flexneri, Salmonella senftenberg, and Arizona arizonae), equivalent respiration was observed (Fig. 15). These data indicate that both pathogenic as well as nonpathogenic bacteria could use substrates that were present in the river water and those sorbed on the bottom sediments after elution by relatively mild laboratory treatment.

Utilization of substrates in detritus samples: The data in Table 28 indicates that coliform bacteria can metabolize substrates present in mildly concentrated detritus from the Oconee River. It is interesting to note that E. aerogenes, an organism associated with soil, can metabolize the detritus at a faster rate than E. coli (0.02-0.03 to 0.00-0.02 mg O/mg dried cell weight/g hexose/hr.) These data and those obtained with river water and stream sediment eluates suggest that while both E. coli and E. aerogenes can metabolize substrates present in the aquatic environment, E. aerogenes would be more successful in competition.

Part 3

Evaluation of the Ability of Enteric Bacteria to Use Natural Aquatic Substrates by Continuous Culture

Preliminary experiments with native populations

Bacterial attachment studies: From the data obtained in the water quality studies (Tables 1-19, 29) and in the respiration experiments (Tables 24-28, Fig. 14), it was concluded that initial experiments to demonstrate bacterial growth in river water could best be accomplished with a natural bacterial population and river water from site 3. These experiments were designed around the incubation temperatures of 14 and 26 C; 14 C being the mean of the water temperature in the fall and spring, and 26 C occurring during the summer. For these experiments the dilution rate was arbitrarily set at 0.058 hr^{-1}.

Figure 16 shows the results of a 14 C experiment. An initial drop in the suspended organisms was observed, and this was attributed mainly to organisms leaving the suspended population and attaching to the glass surfaces. The organisms then decreased sharply and, at nine hours again reached a concentration comparable to that of the culture vessel. These data indicate that the organisms were attaching to the glass, multiplying and releasing cells into the suspension for subsequent wash-

out. Although the enterics and coliforms actively grew and metabolized, the proportion of these populations in the culture diminished with respect to time.

Figure 17 shows the results of an experiment run at 26 C. There appeared little difference between the percentage of attached enterics and coliforms and those in suspension, but the entire culture seemed to fluctuate more than was observed at 14 C. As predicted, growth and attachment of organisms in the culture vessel occurred much more rapidly at the higher temperature.

Analysis of the basal nutrient concentration data from these experiments is difficult since the precision of the tests does not allow for the detection of very small differences in concentration. In general, though, there was a decrease in hexose and ammonia nitrogen in these experiments at both incubation temperatures, little change in phosphate and an increase in protein concentration, and these data are indicative of a metabolizing culture and agrees with data by Herbert, et al. (1956).

These results and those of Sanders (1966, 1967) demonstrate that bacterial attachment to the glass surfaces in a continuous culture device is an important consideration where the results of the suspended population are analyzed. However, such data, as evidenced by the coliforms in our system, suggest that attachment may be a mechanism by which low growth-rage organisms manage to contribute significantly to the culture. This group of slow-growing organisms, in the attached state, could account for otherwise puzzling increased in the suspended population that might be labeled as "bursts" of growth.

Bacterial growth studies: The growth of native bacterial populations in dilute nutrient systems can be clearly shown if longer termed experiments are employed than in the attachment studies. Data in Figs. 18 and 19 demonstrate this observation. Figure 19 shows the results of an experiment run with dilute minimal salts-glucose medium (Dilution rate = 0.012 hr^{-1} at 30 C). With the exception of the coliform bacteria counted at 30 C (upper panel), all groups of organisms increased in number during the first 24 hrs of growth. After that time, the various populations of organisms grew at a rate equal to or greater than the flow rate (Table 30) but began to oscillate with periodicity of about 100 hrs. The fecal coliforms (estimated by the 44.5 C coliform counts in the lower panel) in this system were not as competitive as the other populations and these data suggest that they would eventually be washed out of the system.

When river water from site 3 was substituted for dilute minimal medium as a nutrient source, it was observed that the population of organisms counted at 30 C also increased in number during the first 24 hrs of growth (Fig. 18). After that time, the population decreased in magnitude but yet demonstrated a positive growth rate (Table 30). The coliforms and enteric populations at 44.5 C did not grow but rather, died at a rate faster than the washout rate; however, the heterotrophic group of organisms at this temperature were by far the best competitors. It is interesting to note that by the 420th hour of the experiment, the population counted at 30 C was approximately equal in magnitude to the heterotrophic population counted at 44.5 C.

Oscillations with approximately a 100 hr periodicity were also observed in the river water experiments, and they seemed much more severe than those observed with the dilute minimal medium experiments. Explanations for the periodicity in these experiments are difficult, but it is possible that those organisms which have attached to the glass surfaces could be sloughed off at particular times. Sanders (1966) has shown that the bacterial slime in chemostats can be sloughed off if the underlying portions go sufficiently anaerobic, and even though these experiments were constantly aerated, the system was depleated with oxygen rapidly after initiation of an experiment.

Continuous culture experiments using the stock enteric cultures with river water and collected detritus

River water experiments: River water from sites 1 and 2 would not support the growth of any of the six test bacterial species used. Representative data for E. coli at 30 C is summarized in Fig. 20. Water from site 3 (below the sewage plant effluent) did, however, support the growth of all six strains, including pathogens, at 30 C. Growth of the test organisms at 20 and 5 C was sparse or non-existent. Although the growth rates achieved at 30 C are small and probably not significant as compared to those achieved with full-strength laboratory media, they are comparable to those reported by Jannasch (1969) for Achromobacter in seawater. These data reported here suggest that some enteric species including pathogens are capable of growth in river water at temperatures representative of the environment.

Detritus studies: Attempts to demonstrate growth of the enteric bacterial strains in the detritus samples were generally unsuccessful. Table 32 does, however, contain data for E. coli in continuous culture (Dilution rate = 0.012 hr^{-1}) with concentrated detritus as the nutrient source. The observed growth rate of 0.009 hr^{-1} is low

compared to that which can be achieved in water alone (0.029 hr^{-1}). By working with the hypothesis that a significant portion of the nutrients present in the concentrated detritus has sorbed to contaminating sands and clays in the sample, it was demonstrated that nutrients could be made available by elution of 0.3 M phosphate buffer as was shown in the respiration studies (Table 33).

Bdellovibrio bacteriovorus-Escherichia coli interactions in continuous culture

Activity in river water: After demonstrating that the B. bacteriovorus recovered from the Oconee River will feed upon the stocked E. coli used in the continuous culture experiments (Fig. 21), it was observed that the bacterial parasite was also active in static cultures of river water that was either sterilized by membrane filtration or in the autoclave from site 3 (Fig. 22). It was also demonstrated (Fig. 23) that Bd. bacteriovorus activity could be stimulated if nutrients were added to the system, but numbers of infecting Bd. bacteriovorus would not keep pace with the developing E. coli population. These results indicate that, while the Bdellovibrio population remains viable in river water and capable of responding to situations where the growth of the host organism is favored, the bacterial parasite is unable to successfully eliminate or drastically reduce the host population. This observation is further substantiated by the continuous culture data in Fig. 24 which indicated that an increasing portion of Bdellovibrio population capable of attacking the E. coli host is lost by the 350th hour of the experiment. Reasons for this loss in population are speculative, but either a development of resistance on the part of either the host or the parasite could account for the loss. The latter hypothesis favored because a third population of a yellow colony forming organism was beginning to develop within the chemostat at this time which other investigators have shown to be a heterotrophic form of the Bdellovibrio bacteriovorus (Stolp and Starr, 1963; Shilo and Bruff, 1965, Simpson and Robinson, 1968).

The experiments in this investigation strongly suggest that more research is needed to fully elucidate the interaction between micro-organisms and the underlying sediment portion of the stream. Although these data cannot be directly extrapolated to give information on the fresh water ecosystem, a model can be proposed which suggests bottom sediments can control the nutrient material which is suspended in the water. Work by Wright and Hobbie (1965) has shown the rate at which nutrients turn over to be significant for sustaining heterotrophic

bacterial growth in lake water, and Hendricks and Morrison (1967a, b) have speculated that only a minor component of the total bacterial growth and reproduction can probably occur within free flowing water of a high-quality stream since nutrients are normally in low concentration. However, estimations of nutrient concentration and numbers of organisms are not constant within fresh or marine environments and may vary considerably from time to time. The observations (Morrison and Fair, 1966; Low et al. 1968; Anthony, 1970) can be explained on the basis that a portion of the sediment-nutrient complex can be removed by aqueous extraction which could then be resuspended or dissolved in the water. As the apparent nutrient concentration increased in the river water, increased heterotrophic bacterial growth might also be observed. However, once the adsorptive capacity of the sediments has been reached, as perhaps exists around sewage plant effluents, stream nutrients then could not be removed from the system and much growth of aquatic organisms could result. An occurrence such as this would seriously affect the aerobic component of the system and lead to an altered self-purification potential for some distance below a sewage outfall.

In summary, the presence of coliform bacteria in water for domestic consumption is often considered as evidence for fecal contamination by public health authorities. These organisms, while they primarily do not produce intestinal disease, do serve as indicators for potentially infectious microorganisms. Since these bacteria can gain access to water supplies by a variety of means, any growth by either the coliform group of bacteria or the disease producing organisms in the natural aquatic environment could significantly alter our present concepts of the detection and surveillance of these organisms.

Rivers and streams are constantly involved in a process of partial self purification in which complex materials are broken down by microorganisms for later reuse by other organisms. While most of the nutrient material is present in the water for subsequent utilization, a significant portion is trapped by the sands and clays forming the stream bottom in many areas.

Using tests which are involved in detecting and analyzing polluted waters and from levels of the self-purification potential, we have found that water of the Oconee River, a typically non-polluted stream of the North Georgia piedmont, is capable of supporting the growth of bacteria including coliforms. We have also found that both organic and inorganic compounds can be adsorbed by stream bottom sediments and some of these compounds after removal can be then used as food

for both coliform and intestinal disease producing bacteria.

The significance of our research is two fold and the immediate problem is of a public health nature. If intestinal disease producing bacteria such as Salmonella, Shigella, and Arizona can gain access to our streams and multiply, then there are increasing chances of human disease from the untreated or partially treated water. The long range implications are potentially more serious. Pollution of water resources by partially treated sewage, agricultural and certain industrial wastes and wastes of wild or domestic animals feeding along drainage areas, not only can contribute bacteria but also nutrient material that both the coliform and disease producing enteric bacteria can use for growth which then would increase any immediate public health problem that might exist.

Since the presence of coliform bacteria in water is only indicative of fecal pollution, any increase in numbers of these organisms above a minimal level might necessitate expensive additional treatment or cause rejection of the water for a particular purpose. Substantial changes in our laboratory differentiation procedures for the identification of harmful bacteria in water supplies, and changes in our thinking are going to be required in the coming decade if we are going to best use our aquatic resources.

SECTION VII

ACKNOWLEDGEMENTS

Much of the laboratory work in this investigation was performed by Martha Fleeman, Susan Cubillas, Gay Walter, Flossie Bonner, and Cynthia Carr, while Doyle Anderson, David Lewis, David Mize and Tom Fisher participated in the field studies. Their help is gratefully acknowledged.

Valuable assistance has been rendered by Allen Cherer, Marcia Durso and Stephanie Sanders who each worked on portions of this investigation and received a stipend from the NSF-SSTP program.

The support of the project by the Environmental Protection Agency and the help provided by Pat C. Kerr of the Southeastern Water Laboratory, and by Drs. Walter M. Sanders and Russell L. Todd, the Grant Project Officers, is acknowledged with sincere thanks.

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SECTION IX

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2. Cubillas, Susan, and Charles W. Hendricks. 1970. Aquatic bacterial respiration in a continuous culture system. Bacteriol. Proc. p. 32.

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Patents: None, but either one or both of the chemostats could be patented.

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SECTION X

GLOSSARY

Basal Nutrients - A combination of simple inorganic and organic compounds that will support the growth of prototrophic bacteria.

Biotope - A place of life; the totality of the environmental conditions under which a community of organisms exist.

Chemosynthetic - Organisms that synthesize organic matter from mineral or organic substances with the aid of chemical energy.

Detritus - Finely divided settleable material suspended in the water.

Dilution Rate - The rate at which nutrients are added to a continuous culture device, and wastes are removed from the system.

Enzyme induction - The initiation of enzyme synthesis within an organism.

Heterotrophic - The nutrition of plants and animals that are dependent on organic matter for food.

Mesophilic - A mid-range temperature requiring organism (20-45 C).

M - Moles or Molar

μ - Micro or Micron

Pathogenic - The ability of an organism to produce disease or infections.

Photosynthetic - Organisms that produce organic matter from CO_2 and H_2O with the aid of the energy of light.

Protrotrophic - The ability of organisms to grow on mixture of inorganic salts, water, and a simple carbohydrate (i. e. glucose minimal medium).

Substrate - A chemical compound that is suitable for metabolism by an organism.

U. S. GOVERNMENT PRINTING OFFICE : 1972—484-484/179

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